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ALBUMIN EXCRETION IN DIABETES MELLITUS

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DECLARATION

The work detailed in this thesis was undertaken in the Department of Medicine, University of Newcastle upon Tyne from January 1983 to September 1988. All of the work was performed by myself apart from the following:

1. The albumin and β_2 -microglobulin measurements in Chapters 7 and 10 were performed by Mrs Pat Shearing.
2. Dr Sam Dagogo-Jack determined the epidermal growth factor concentrations.
3. Dr MF Laker, Mr R Manuel and Mr K Harrison measured glycosylated haemoglobin, fructosamine and urine and serum creatinine.

SUMMARY

Diabetes mellitus carries a twenty-fold excess mortality for insulin-dependent (IDDM) and two-fold for non-insulin dependent (NIDDM) patients compared with the non-diabetic population. Almost all of this excess is confined to those people who develop persistent proteinuria and is due mainly to cardiovascular and renal disease. In insulin-dependent diabetes, uraemia is common, whilst in non-insulin dependent diabetes, cardiovascular disease accounts for the majority of deaths. Once persistent proteinuria develops, progression of IDDM patients to end-stage renal disease is inevitable.

The dip-stick tests classically used to detect proteinuria are insensitive. Sensitive immunoassay techniques have shown that urine from healthy subjects normally contains <20 mg albumin/l. There is thus a large gap between the normal range and pathological proteinuria detected by conventional testing. Diabetic patients may excrete more albumin than normal subjects, but remain dip-stick negative - so called microalbuminuria. Several studies have shown that those IDDM patients with microalbuminuria are the ones who progress to persistent proteinuria, whilst in non-insulin-dependent diabetes, similar levels of albumin excretion are predictive of early death, mainly from cardiovascular disease. The cut-off point of microalbuminuria predicting increased risk is 30 $\mu\text{g}/\text{min}$ in a timed overnight urine collection.

A sensitive radioimmunoassay for urinary albumin has been established and validated. The albumin excretion rate (AER) in timed overnight samples in 106 healthy adults was 5.4 (1.7-10.5) $\mu\text{g}/\text{min}$ (geometric mean and range). The excretion rate was higher in males than females [5.8(2.0-11.2) v 3.6(1.4-9.3) $\mu\text{g}/\text{min}$; $p<0.005$]. No influence of age on AER was seen. In 64 healthy children mean age 9 years (range 3-17), AER was significantly lower than in the adult population [2.5(0.6-7.0) $\mu\text{g}/\text{min}$; $p<0.001$]. Excretion rates were similar in boys and girls, but AER correlated with age ($r=0.33$, $p<0.001$).

AER was measured in 940 unselected diabetic patients, 416 IDDM and 524 NIDDM. Seventy one percent had AER in the normal range, 15% had AER between 10.6-29.9 $\mu\text{g}/\text{min}$, 8.4% 30-150 $\mu\text{g}/\text{min}$ and 5.5% >150 $\mu\text{g}/\text{min}$. The mean AER was higher in the NIDDM patients ($p<0.01$) and the proportion of NIDDM patients with abnormal rates of albumin excretion was higher than in IDDM ($p<0.002$). Univariate analysis showed that in IDDM, AER was related to known duration of diabetes ($r=0.21$,

$p < 0.001$), systolic BP ($r = 0.18$, $p < 0.001$), male sex ($t = 2.46$, $p < 0.002$) and the presence of retinopathy ($t = 4.61$, $p < 0.001$), peripheral vascular disease ($t = 2.85$, $p < 0.01$) and peripheral neuropathy ($t = 2.50$, $p < 0.02$). On multiple regression analysis the presence of retinopathy and male sex remained significant ($r = 0.28$, $p < 0.001$).

In NIDDM, AER correlated with known duration of diabetes ($r = 0.18$, $p < 0.01$), systolic ($r = 0.20$, $p < 0.001$) and diastolic blood pressure ($r = 0.17$, $p < 0.001$) and to the presence of peripheral vascular disease ($t = 4.12$, $p < 0.001$), retinopathy ($t = 3.66$, $p < 0.001$) and ischaemic heart disease ($t = 2.64$, $p < 0.002$). On multiple regression analysis, systolic blood pressure, peripheral vascular disease and retinopathy remained significant ($r = 0.31$, $p < 0.001$). In IDDM, only one patient with duration of diabetes less than 5 years had AER 30-150 $\mu\text{g}/\text{min}$, whereas this level was observed at all durations of NIDDM, presumably because of the long silent phase of NIDDM.

Albumin excretion was measured in 68 children with insulin-dependent diabetes, with mean duration of diabetes 3.8 (range 1-13) years. AER was similar to that of non-diabetic children [2.5(0.7-9.5) v 2.4(0.2-20.8) $\mu\text{g}/\text{min}$] and correlated with age ($r = 0.36$, $p < 0.001$). When matched for age, there was no difference in AER between diabetic boys and girls [3.1(0.9-9.5) v 2.3(1.1-7.5) $\mu\text{g}/\text{min}$].

Day-to-day variation in albumin excretion is high. In 8 healthy adults, the coefficient of variation (CV) of AER measured on 9.4 (range 7-11) occasions over 6 weeks, was 13.1%. In diabetic patients the CV was 47.2% in those with normal AER, 17.0% in microalbuminuric patients and 10.5% in those with persistent proteinuria. During follow-up for at least 18 months the mean rate of change of AER was +11.3(-31.4 - +105.5)% per year in normoalbuminuric patients and +1.3(-44.4 - +101.8)% per year in microalbuminuric patients. Several patients showed large fluctuations in AER.

The predictive power of microalbuminuria is based on albumin excretion rates measured in timed urine collections, which are impractical and open to many sources of error. Measurement of albumin concentration on an aliquot of overnight urine had poor sensitivity and specificity in identifying those patients with AER 30-150 $\mu\text{g}/\text{min}$. However, correction for creatinine excretion improved the value of the test considerably. Thus, an albumin:creatinine ratio > 3.5 had a sensitivity of 98% and specificity of 69% in predicting AER 30-150 $\mu\text{g}/\text{min}$. Measurement of

albumin:creatinine ratio in an aliquot of the first morning urine may thus be an acceptable screening test for microalbuminuria.

It is likely that the detection and monitoring of abnormal albumin excretion will become routine in the diabetic clinic. Microbumintest is a commercial reagent tablet which the manufacturers claim will detect albumin concentrations $>40 \mu\text{g/ml}$. Evaluation of the tablet by 1 operator using 106 urine samples [albumin concentration $34.5(1.5-149) \mu\text{g/ml}$; mean (range)] revealed a sensitivity of 84% and specificity 80%. Eight operators tested 24 urine samples twice. There was a large intra- and inter-operator variability. This variability, plus the low sensitivity and specificity, make Microbumintest unsuitable for routine use.

Urin-Pak is a commercially marketed, automated immunoturbidimetric method of albumin measurement. When compared with an in-house radioimmunoassay, the overall correlation was good ($r=0.91$, $y=1.02x+0.078$), precision satisfactory (intra- and inter-assay CV at albumin concentration of $40.7 \mu\text{g/ml}$, 5.2%) and the method proved easy and quick to use. However, 28% of the samples lay outwith $\pm 20\%$ of the line of identity.

The hallmark of diabetic nephropathy is the accumulation of abnormal mucopolysaccharide material in the mesangium and basement membrane. The mucolytic agent bromhexine decreases glomerular volume in experimental diabetes and also decreases the urinary excretion of mucopolysaccharides in IDDM. In a randomised, double-blind, placebo-controlled trial, bromhexine 72 mg daily had no effect on albumin excretion in IDDM patients with normoalbuminuria, microalbuminuria and persistent proteinuria.

Growth factors may be involved in diabetic renal hypertrophy. The role of epidermal growth factor (hEGF) has been examined. Albumin and hEGF excretion were measured in 19 healthy adults with normal albumin excretion and 55 diabetic subjects with AER $1.4-879 \mu\text{g/min}$. The hEGF:creatinine ratio was decreased in the diabetic subjects with abnormal albumin excretion. There was a significant correlation between hEGF:creatinine and AER ($r=-0.49$, $p=0.02$).

Thus abnormal albumin excretion is common in diabetes and reflects widespread vascular disease. Identification of patients with abnormal excretion by frequent testing over many years, coupled to aggressive management of the associated abnormalities, may lead to diminution of the excess mortality carried by these patients.

CHAPTER 1

Introduction

1.1 The excess mortality associated with diabetes mellitus

It was not until many years after the introduction of insulin treatment for diabetes mellitus in 1922 that the full ravages of the long-term complications of diabetes became clear(1,2,3,4,5). In 1953 Lundbaek published the results of a survey of 234 patients who were found to have diabetes between 1924 and 1934 and who had survived for at least 15 years (1). Thirty percent had died, the common causes of death being heart disease, cerebrovascular disease and renal insufficiency. Significant numbers of survivors had evidence of retinopathy, nephropathy and coronary and peripheral vascular disease. Lundbaek concluded that the various long-term manifestations of diabetes were different expressions of a generalised, specific diabetic vascular disease.

In a study of 317 Danish patients diagnosed diabetic before the age of 31 years and before 1933, the mortality rate after 40 years of diabetes was 2-6 times that of an age and sex matched non-diabetic population(5). Uraemia was the cause of death in 31% of cases and myocardial infarction in 25%. In an American study of 1966 children found to have insulin-dependent diabetes (IDDM) before the age of 17 years, and diagnosed between 1950 and 1981, a sevenfold excess in mortality was found(6). After the age of 20, the annual mortality rate was 20 times that of the non-diabetic population. The cause of death was renal disease in almost 40%, with cardiovascular disease accounting for a further 10%. A UK survey of deaths occurring during 1979 in diabetic patients under the age of 50 years, found that 15% of 447 deaths were from nephropathy(7). In those patients diagnosed before the age of 31 years, almost equal numbers died of renal and cardiac disease which together accounted for over half the deaths. Death in IDDM patients with nephropathy is attributed to renal failure in approximately 60% and cardiovascular causes in 24-36% (7,8,9).

In non-insulin dependent diabetes (NIDDM), such clear-cut data is not available, but several studies have found an overall mortality rate of twice that of the non-diabetic population(3,10). The major cause of death is cardiovascular disease, being responsible for 60% of deaths, with renal disease accounting for only relatively few deaths(10,11,12). In the USA, the relative risk of renal mortality in diabetic patients aged over 45 years is doubled, compared with the twenty-fold excess seen in those

under 45 years(13). In the UK in 1983-84, 24% of the diabetic patients accepted for renal replacement therapy had not been treated with insulin(14). Thus, although the risk to the individual patient is small, because of the high prevalence of NIDDM, the number of patients with NIDDM who may potentially develop diabetic nephropathy is large.

Thus both IDDM and NIDDM carry a considerable risk of early mortality, particularly from renal and cardiovascular disease. In IDDM, renal disease predominates, whilst in NIDDM, cardiovascular disease is more common.

1.2 Proteinuria and excess mortality

The importance of proteinuria was recognised early. In 1936 Kimmelstiel and Wilson described 8 patients with the clinical syndrome of oedema of the nephrotic type, renal decompensation, hypertension and large quantities of albuminuria, who at autopsy had the classical histological features of diabetic nephropathy(15). In a very large study of 1465 autopsies performed on diabetic subjects, Bell highlighted the importance of severe proteinuria in suggesting the presence of advanced glomerular lesions, particularly in young subjects(16). The importance of proteinuria as a predictor of excess total mortality and not only of renal disease has been demonstrated in several studies. In the Danish study of young patients diagnosed before 1933, the excess mortality among patients who developed persistent proteinuria within 40 years of diagnosis of diabetes was 3-4 times higher than in patients who did not have proteinuria after 40 years(5). In the cohort diagnosed before 1953, proteinuria developed in 41%(8). After 40 years of diabetes, only 10% of those who developed persistent proteinuria were alive, whereas more than 70% who did not develop proteinuria survived. In an almost identical group of patients, Borch-Johnsen showed that the relative mortality in those without proteinuria remained constant at about 2, whilst for those with proteinuria, there was a dramatic increase in relative mortality to a maximum of 100 at age 35 years(17). Conversely, in a group of 92 patients who had had diabetes for more than 40 years, only 8 had proteinuria(18).

It would thus seem that in IDDM proteinuria is a marker for those patients at increased risk of early death from both renal and cardiovascular causes. The significance of proteinuria is less well studied in NIDDM, although in a small study an excess mortality was found in those with marked proteinuria(19).

1.3 The inevitable progression from persistent proteinuria to end-stage renal failure

In IDDM, once persistent proteinuria develops, the decline in renal function is relentless, the interval to end-stage renal failure averaging 7-10 years with a range of 1-25 years(8,9,20,21). No therapeutic intervention yet tried has halted or reversed the decline. All patients with declining renal function have hypertension(7,9,20,22) and patients with persistent proteinuria but normal serum creatinine have significantly higher blood pressures than matched diabetic patients with normal albumin excretion and normal controls(23). Elevated blood pressure may accelerate the decline in renal function in animals(24) and in man(25). Antihypertensive therapy (β -blocker, diuretic, hydralazine) has been shown to decrease the rate of decline of glomerular filtration rate (GFR) but not to halt the progression(26,27,28,29).

Improved blood glucose control using continuous subcutaneous insulin infusion (CSII) was tried for up to 24 months in 6 patients with persistent proteinuria, with no effect on the mean rate of decline of GFR or fractional clearance of albumin(30). However, numbers were small, blood glucose control although improved was not normalised, and there was considerable variation in response between patients. Thus whilst a dramatic effect on the decline in GFR probably does not occur with improving blood glucose control, a small effect in individual patients may have been missed. At the slightly earlier stage of intermittent proteinuria, improved blood glucose control has also been demonstrated to have no effect on GFR, although this study was marred by the wide variation in albumin excretion rates in the patients studied(31).

Dietary protein restriction has also been studied as a means of slowing the decline in renal function. In animals(32,33) and in normal man(34,35) protein ingestion increases GFR. It has been suggested that hyperfiltration may be involved in the pathogenesis of renal disease(36) and that a raised GFR may predict the future development of diabetic nephropathy(37). An acute (3 week) trial of protein restriction (to around 40g/day) in patients with diabetic nephropathy showed a fall in the fractional clearance of albumin and IgG but no change in GFR(38). However, preliminary reports on a long-term (> 1 year) study suggest that decreased dietary protein intake to around 45g/day in IDDM patients may reduce the rate of decline of GFR significantly(39).

Thus it is clear that after the development of persistent proteinuria, the decline to end-stage renal failure is relentless, intervention at best only slowing the rate of decline. Whether it is possible to alter the risk of cardiovascular disease also carried by patients

with persistent proteinuria has not been studied but it seems unlikely that at this advanced stage any dramatic effect would be seen. Prevention of diabetic nephropathy and perhaps also cardiovascular disease must thus take place at an earlier stage of diabetes, before the development of persistent proteinuria. The problem of how to identify those patients who would later develop proteinuria then arose.

1.4 Microalbuminuria

Persistent proteinuria as described above is generally defined as the excretion of more than 0.5g protein/24h in at least 3 consecutive urine samples collected over 1 year. Testing for protein has been by semiquantitative means with poor sensitivities such as dip-stick testing (Albustix, Ames Limited, Stoke Poges, Slough, UK) or by crude laboratory techniques such as the sulphosalicylic acid test. In 1963, Keen and Chlouverakis described a sensitive radioimmunoassay for urine albumin measurement (40). They later demonstrated that in a 2h ambulant period, the mean albumin excretion was 3.4mg in normal controls and significantly higher at 4.9mg in newly diagnosed diabetic subjects, mainly NIDDM(41). A similar elevation in albumin excretion rate (AER) was seen in 5 newly diagnosed IDDM patients, the AER falling to normal 14 days after the start of insulin treatment(42). In this same paper, AER was measured in 22 IDDM patients without persistent proteinuria, with duration of diabetes ranging from 1-40 years. Daytime AER ranged from 3.9-96.3 $\mu\text{g}/\text{min}$, compared with a reference range of 5.8-22.6 $\mu\text{g}/\text{min}$ (42). In IDDM patients with a short duration of diabetes, Parving showed a significant rise in AER after withdrawal of insulin therapy(43). In another group of 80 non-proteinuric IDDM patients with diabetes duration 1-39 years, elevated AER was found in 41%(44). It was thus obvious that diabetic subjects without persistent proteinuria could exhibit increased albumin excretion above the normal range. Such a level of albumin excretion has been called microalbuminuria.

The full significance of microalbuminuria was revealed in 1982 when several studies were published describing the fate of those patients who had previously shown elevated levels of albumin excretion. In 1966-67, the Guy's Hospital group measured overnight AER in 87 IDDM patients without persistent proteinuria. At follow-up 14 years later, information on 63 of the original cohort was available. Of the 8 who initially had an AER $>30 \mu\text{g}/\text{min}$, 7 had developed persistent proteinuria and 3 (37.5%) were dead. Of those with an initial AER $<30 \mu\text{g}/\text{min}$, 2 out of 55 had developed proteinuria and 5

(9.1%) were dead(45). It thus appeared that an AER $>30 \mu\text{g}/\text{min}$ was predictive of persistent proteinuria and increased mortality. Several papers confirming this report quickly followed(37,46,47). They are summarised in Table 1. Although the types of urine collection, albumin assay, length of follow-up and cut-off for AER are different in each study, the conclusion in each paper is similar, ie that IDDM patients with an AER above a certain level are those who will later develop persistent proteinuria. Overall, of the 37 patients with an initially "high" AER, 32 developed persistent proteinuria, whilst only 7 of 163 with initial "low" AER progressed.

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These observations were later extended to NIDDM patients. Jarrett studied 42 NIDDM patients, with urine negative to Albustix, initially in 1966-67 and again in 1980. Of the 25 survivors, 24 had initial AER $<10 \mu\text{g}/\text{min}$, whilst of the 17 deceased, 6 had initial AER $>31 \mu\text{g}/\text{min}$ and 10 $>10 \mu\text{g}/\text{min}$ (48). The mortality risk for subjects with AER >31 or $>10 \mu\text{g}/\text{min}$ was 3.3 and 4.0 respectively. In a larger study with a 10 year follow-up, the increased mortality of NIDDM patients with an initial albumin concentration of $30 \mu\text{g}/\text{ml}$ in a morning urine sample was confirmed and shown to be due mainly to cardiovascular disease(19). In this study, these patients were also at risk of progression to persistent proteinuria.

It would thus seem that in both IDDM and NIDDM, increased albumin excretion above normal but not yet in the Albustix-positive range carries a risk of progression to persistent proteinuria and also of early death from cardiovascular disease in NIDDM. It is noteworthy that in several papers, there is a group of diabetic subjects with AER clearly elevated above normal but who have not been shown to be at risk(37,45,47). Thus in the Guy's Hospital paper, those patients with AER $12\text{--}29.9 \mu\text{g}/\text{min}$ have albumin excretion outwith the normal range but have not been included in the at-risk group(45). The different levels of AER used to divide the groups in the 6 papers described above indicate that the precise level of AER at which the risk is increased is unclear; nor are the best methods of determining AER e.g. type of urine collection, albumin assay methods, understood. The follow-up period in the studies ranged from 6-14 years. Hence we have no clear idea of the rate of progression of microalbuminuria to persistent proteinuria. Despite these deficiencies, we now appear to have a reliable means of identifying much earlier in the course of their disease, those patients at increased risk of diabetic nephropathy and early death. This observation opens the way to trials of therapeutic intervention at an early stage.

	Viberti(45)	Parving(46)	Mogensen(37)	Mathiesen(47)
Number of patients	63	23	43	71
Length of follow-up (years)	14	6	>7	6
Type of urine sample	overnight	24h	short day time	24h
Number of samples	≥1	1	≥3	1
Albumin assay	RIA	RID	RIA	RID
Normal range AER	≤12μg/min	≤40mg/24h	≤7.5μg/min	≤20μg/min
Discrimination AER	30μg/min	40mg/24h	15μg/min	70μg/min
Number above discriminant level developing proteinuria	7/8	6/8	12/14	7/7
Number below discriminant level developing proteinuria	2/55	2/15	0/29	3/64
RIA: radioimmunoassay				
RID: radial immunodiffusion				

Table 1 Summary of the studies showing the predictive power of microalbuminuria in insulin dependent diabetes

1.5 Albumin excretion in normal subjects

In most papers describing abnormal albumin excretion in the diabetic population, comparison has been made with only small numbers of normal subjects(42,44,46). Only in the Bedford study were large numbers of non-diabetic subjects investigated but here albumin excretion was measured in a very special situation, during a 50g oral glucose tolerance test with the subjects remaining ambulant(41). It is obviously important to establish reference ranges for AER in a large population of normal subjects under standard conditions of urine collection and laboratory analysis, and to examine the effect of age and sex on AER. AER has been measured in 106 normal adults and 64 children. The results are given in Chapter 3.

1.6 Comparison of the prevalence and associated features of abnormal albumin excretion in insulin-dependent and non-insulin dependent diabetes

Identification of those patients at risk of early death or of end-stage renal disease is obviously important to allow therapeutic intervention and also to allow more accurate planning for facilities for later treatment. The initial work on microalbuminuria was done in departments with a declared interest in diabetic nephropathy, so no reliable figures as to the prevalence of microalbuminuria in an unselected population are available. Additionally, the prevalence of microalbuminuria in IDDM and NIDDM has not been compared.

Although several studies have reported a very high incidence of persistent proteinuria in IDDM(6,8,17) and NIDDM(49), the current prevalence of persistent proteinuria in the UK diabetic population seems very variable. In a large study of an apparently unselected out-patient population, only 2.7% had proteinuria exceeding 0.2g/24h(50). However, in another study of insulin-treated patients, 9.4% had persistent proteinuria by Albustix testing and 5.2% intermittent proteinuria(51). Although these 2 studies cannot be strictly compared, the large difference warrants further investigation.

As we have already seen, blood pressure is already elevated at the stage of persistent proteinuria with normal serum creatinine concentration(23) and hypertension is a well-recognised feature of end-stage renal disease(7,20,22). It is thus of interest to determine the blood pressure level in patients with microalbuminuria.

Retinopathy(7,20) hypertension (7,20,22,23) and male sex(8,12,17) have long been associated with end-stage renal disease in IDDM. Whether these features are also seen in nephropathy in NIDDM is unclear. It would also be of interest to know if these associations are seen in patients with microalbuminuria. As the relative risks of renal and cardiovascular disease in IDDM and NIDDM patients with microalbuminuria appear to be different it might be expected that the features associated with microalbuminuria in the 2 types of diabetes might be different.

In chapter 4, the prevalence of the various degrees of albuminuria in an unselected clinic population of NIDDM and IDDM have been compared and the relationship of albumin excretion to age, sex, duration of diabetes, blood pressure and the presence of the other complications of diabetes explored.

1.7 Albumin excretion in children with diabetes and its relation to clinical variables

There have been no longitudinal studies of albumin excretion in children showing that those with microalbuminuria are at risk of progression to diabetic nephropathy as in adults. However, there seems no *a priori* reason why they should not be. Indeed, one study has suggested that relative mortality in IDDM increases with decreasing age at diagnosis(52). Several studies have found increased albumin excretion in diabetic as compared to normal children(53,54,55,56) whilst one has not(57). Additionally there have been discrepancies in the effect of age on albumin excretion. A comparison of albumin excretion rates in normal adults and children and children with diabetes may be of some help in deciding where the cut-off for at-risk microalbuminuria in children might lie.

The overnight albumin excretion rates in diabetic and normal children and in normal adults have been compared. The relationships of age, duration of diabetes and metabolic control to albumin excretion have been explored.

1.8 The day-to-day and longer-term variation in albumin excretion

A large number of factors are known to affect albumin excretion acutely in normal and diabetic subjects, including posture(42,54,58), exercise(59,60) acute decompensation in metabolic control(42,43), and dietary protein ingestion(61). Thus it seems likely that there will be a large day-to-day variation in albumin excretion

under conditions of every day life. Although several of the studies showing that patients with elevated AER were at risk of nephropathy based their predictions on a single urine sample(45,46,47), a large day-to-day variation in albumin excretion might result in misclassification of individual patients and may also make interpretation of intervention studies difficult if only one measurement of AER were made. The day-to-day variation in AER in normal subjects and diabetic patients has been measured.

As has already been discussed, the rate of progression from persistent proteinuria to end-stage renal failure is very variable, although constant in any one patient. Little information is available on the rate of progression of microalbuminuria to persistent proteinuria, the initial predictive studies having a follow-up interval of 6-14 years(19,37,45,46,47,48).

The rate of progression is obviously important in planning any intervention trial. The AER has therefore been measured in IDDM patients with normal and elevated albumin excretion at regular intervals for several years.

1.9 Mucopolysaccharide deposition and diabetic nephropathy

The early studies of Kimmelstiel and Wilson demonstrated nodular accumulation of hyaline material and basement membrane thickening in the glomerulus(15). With the advent of the electron microscope and percutaneous renal biopsy, the classical histological features of diabetic nephropathy are now known to be thickening of the basement membrane and accumulation of basement membrane-like material in the mesangium(62). Accumulation of this material in the glomerular tuft reduces the available glomerular filtration surface, so that the GFR is highly correlated to the filtration surface per nephron(63). This basement membrane-like material consists of collagen-like glycoprotein(64) and in diabetic subjects is quantitatively abnormal, with a decreased content of heparan sulphate(65). Loss of the anionic heparan sulphate may allow enhanced passage of anionic albumin across the glomerular basement membrane (66).

The vinca alkaloid bromhexine is a mucolytic agent thought to alter glycoprotein synthesis (*Shelley JH, personal communication*). Bromhexine has been shown to decrease the urinary excretion of carbohydrate-containing substances in IDDM(67) and

to decrease glomerular volume and possibly basement membrane thickening in experimental diabetes(68). The effect of bromhexine on albumin excretion in IDDM patients with varying degrees of nephropathy has been studied.

1.10 Epidermal growth factor and diabetic nephropathy

It is well known that at diagnosis of insulin-dependent diabetes, kidney size and GFR are elevated(69,70). After insulin treatment, both fall, although not to normal levels in all patients. A similar increase in kidney size is seen in experimental diabetes shortly after induction of diabetes(71). A sustained elevation of GFR may be important in the development of diabetic nephropathy(37). The causes of the kidney growth and elevated GFR and also of the accumulation of mucopolysaccharide substances described earlier are unclear, but involvement of growth factors is possible.

Epidermal growth factor (EGF) which was first isolated from mouse submaxillary glands(72) and later from human urine(73), is a potent stimulus of the growth of several cell types from a variety of species(74). EGF is synthesised and excreted by the kidney(75,76,77). It is thus a possible candidate as a renal growth factor.

The pattern of hEGF excretion in healthy subjects and in diabetic patients with varying degrees of albuminuria has been examined.

1.11 Screening for microalbuminuria

Given that we now have the means of identifying those diabetic patients at risk of diabetic nephropathy and premature mortality, and that intervention, perhaps in the form of improved blood glucose control, anti-hypertensive therapy or a low protein diet, may decrease that risk, it is likely that screening for microalbuminuria will become appropriate. Large numbers of patients will require to be tested. Since there is a large day-to-day variation in albumin excretion and since we are currently uncertain of the natural history of microalbuminuria, screening will have to be repeated at regular intervals. A suitable screening test will be acceptable and convenient for the patient, easy for the laboratory to hand, have high sensitivity and specificity and be cheap and capable of handling large numbers.

Almost all of the initial papers showing the predictive power of microalbuminuria used timed urine collections - either overnight(45,48), 24h(46,47) or short day-time

collections(37). Timed urine collections are inconvenient for the patient and cumbersome for the laboratory. Errors in timing or failure to collect all the urine, particularly so in a diabetic patient with bladder dysfunction(78) may lead to inaccurate results. Compliance is likely to be poor. Timed urine collections thus do not appear to be suitable as a screening test.

The protein:creatinine ratio in an aliquot of a random(79) or first voided morning urine sample(80) has been shown to correlate well with 24h protein excretion in a variety of renal diseases with heavy proteinuria. Many diabetic patients already bring an aliquot of the early morning urine (EMU) to the diabetic clinic and such a sample would avoid the rise in albumin excretion associated with posture and exercise, which may be exaggerated in diabetes(81,82). The ability of the albumin concentration or albumin:creatinine ratio in an early morning urine sample to predict AER 30-150 ug/min has been examined.

1.12 Biochemical tests for microalbuminuria

As described above, it is likely that screening for microalbuminuria will become a routine requirement for diabetic care. A side-room test, equivalent to dip-stick testing such as Albustix, may be appropriate for initial qualitative screening but for quantification and monitoring the effect of therapy, a sensitive, accurate, reproducible, cheap test capable of handling large numbers is required. Two new methods for detection of low amounts of urinary albumin have been evaluated. Microbumintest (Ames, Stoke Poges, Slough, UK) is a qualitative tablet test designed to detect concentrations of albumin $>40 \mu\text{g/ml}$, perhaps suitable as a screening test performed in the diabetic clinic. Urin-Pak (Ames, Stoke Poges, Slough, UK) is an automated immunoturbidimetric test designed for laboratory use.

1.13 Aims and objectives of this work

The aims and objectives of the work described in this thesis were, therefore, to:

1. Establish and validate a sensitive assay for measurement of urine albumin.
2. Determine the reference range of albumin excretion in normal adults and children and to examine the effects of age and gender on albumin excretion.
3. Determine the prevalence and associated features of abnormal albumin excretion in insulin-dependent and non-insulin dependent diabetic patients.

4. Determine the prevalence and associated features of abnormal albumin excretion in children with IDDM.
5. Examine the day-to-day and long-term variability in albumin excretion in diabetic subjects.
6. Evaluate the albumin:creatinine ratio measured in an early morning urine sample as a predictor of microalbuminuria.
7. Evaluate the commercially-produced kits, Microbumintest and Urin-Pak, for measurement of urinary albumin.
8. Investigate the effect of the mucolytic agent bromhexine on all levels of albumin excretion in IDDM.
9. Investigate the relationship of excretion of epidermal growth factor to albumin excretion in diabetic patients and normal subjects.

CHAPTER 2

GENERAL METHODS

2.1. Albumin Assay

Urine albumin was measured by sensitive radioimmunoassay by a modified method of Christensen and Orskov(83).

a) Iodination

The iodination reaction was carried out in 0.05M sodium dihydrogen phosphate buffer (Na.H₂.PO₄.H₂O), pH 7.5. Human serum albumin, 250 µg in 10 µl, and freshly prepared chloramine T, 10 µg in 10 µl, were added to a vial containing 2 mCi ¹²⁵I. After mixing gently for 30 s, the reaction was halted by the addition of 40 µg in 400 µl sodium metabisulphite. The reaction products were immediately applied to a 1 by 50cm column containing Ultragel AcA 44 (LKB, Bromma, Sweden). Elution was by 0.04M sodium dihydrogen phosphate buffer, pH 8.0, containing 0.2% (w/v) bovine serum albumin (PbB). Approximately 2 ml fractions were collected and 10 µl aliquots counted for 30 s on an NE1600 multi-well gamma counter (Nuclear Enterprises, Edinburgh, UK).

Fractions containing bound iodine were pooled for use in the radioimmunoassay (stock labelled albumin). From the total number of counts in the albumin peak and in the free iodine peak, the specific activity of the labelled albumin and the percent ¹²⁵I bound were calculated thus:

$$\% \text{incorporation } ^{125}\text{I} = \frac{\text{total counts albumin peak}}{\text{total counts albumin peak} + \text{free } ^{125}\text{I}} \times 100$$

$$\text{Specific activity} = \frac{\% \text{incorporation}}{100} ^{125}\text{I} \times 2 \times \frac{1}{250} \text{ } \mu\text{Ci}/\mu\text{g albumin}$$

The incorporation was generally >90%, with specific activities of 6-8 µCi/µg albumin. A typical elution profile of the counts in each fraction is shown in Figure 1. The peak at fraction 31 represents ¹²⁵I bound to albumin and fractions 30 and 31 were pooled to form the stock labelled albumin solution. The shoulder on this peak (fraction 26) represents ¹²⁵I bound to altered albumin and the peak at fraction 57 free ¹²⁵I.

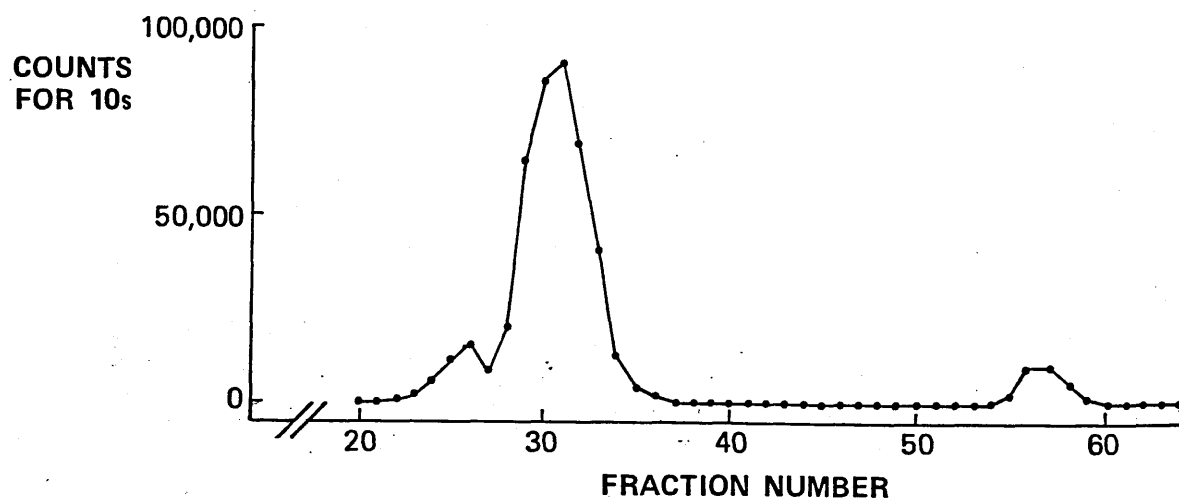


Figure 1. Albumin iodination. Separation of ^{125}I albumin (peak at fraction 31) from ^{125}I altered albumin (fraction 26) and free ^{125}I (peak at fraction 57)

b) Albumin Assay Protocol

The assay was carried out in PbB buffer, pH 8.0. Human serum albumin standards were prepared from 10% human serum albumin (Kabi Diagnostica, Stockholm, Sweden) in concentrations of 0.1-10 µg/ml. Stock labelled albumin was diluted 10 µl in 6 ml to reduce the activity to approximately 30,000 cpm in each tube. Rabbit anti-human albumin immunoglobulin (Dako, Copenhagen, Denmark) was diluted in PbB to a predetermined titre (see antibody binding curve). To 5 µl buffer, standard or sample were added 100 µl diluted label and 100 µl diluted antibody. After mixing, the reaction was incubated at 4°C for 18 hours. Precipitation of bound label was by addition of 500 µl polyethylene glycol 6000, 25% (w/w) in assay buffer and 100 µl donor calf bovine serum (Flow Laboratories, Irvine, Ayrshire, UK). The tubes were mixed and then centrifuged at 2400 rpm at 4°C for 50 minutes. The supernatant was decanted and the precipitate counted on a Packard Auto-Gamma 5780 gamma counter (Packard, Pangbourne, Berkshire, UK). Total counts in each reaction tube were estimated by counting 100 µl label only. Non-specific binding was assessed by precipitated radioactivity in reaction tubes containing 100 µl assay buffer PbB instead of antibody. The binding of label to antibody was checked by inclusion of an "excess" reaction containing 100 µl antibody of titre 1:320 instead of diluted antibody.

Non-specific binding was 5-10% and "excess" binding 92-99% throughout. New labelled albumin was produced every 3 months, or earlier if the non-specific binding rose >10% or "excess" binding fell to <90%. Each standard and unknown was assayed in triplicate.

A typical albumin standard curve is shown in Figure 2. The sensitivity of the assay was 0.5 µg/ml and samples above 5 µg/ml were diluted before re-assay.

c) Antibody Binding Curve

An antibody binding curve was generated for each new batch of labelled albumin or antibody, with antibody diluted in assay buffer over a range of titres from 1:320 to 1:64,000. To 5 µl assay buffer was added 100 µl diluted label and 100 µl diluted antibody. The reaction was completed as described above. A typical antibody binding curve is shown in Figure 3. The titre of antibody used in subsequent assays was chosen to give binding of approximately 60%, commonly 1:32,000.

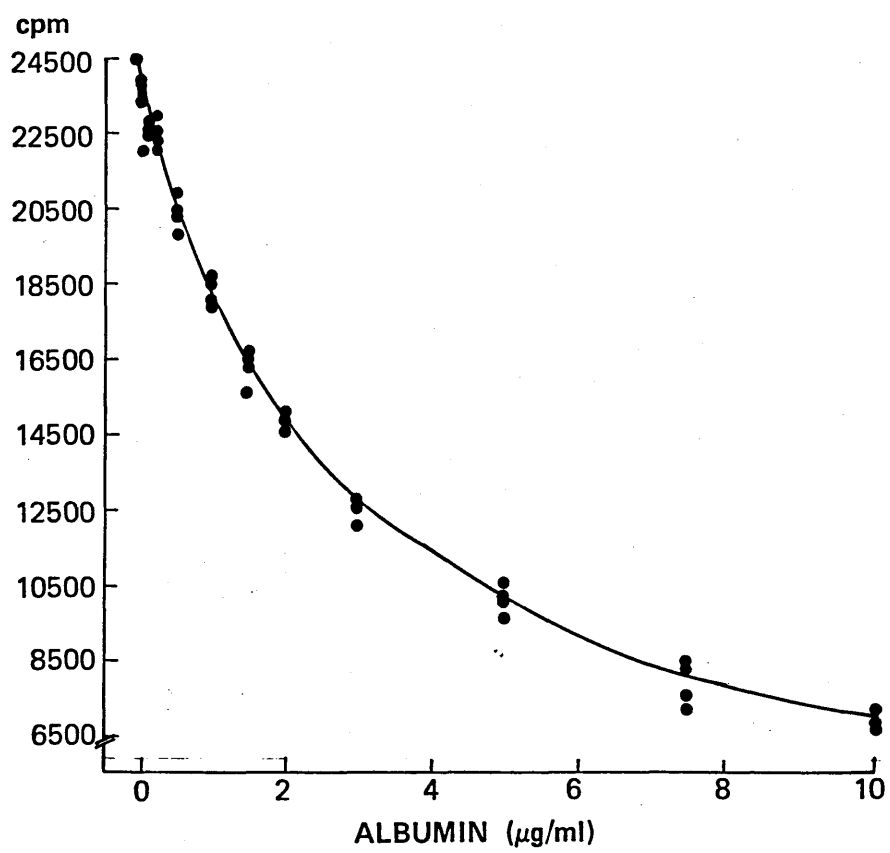


Figure 2. Albumin radioimmunoassay: Standard curve

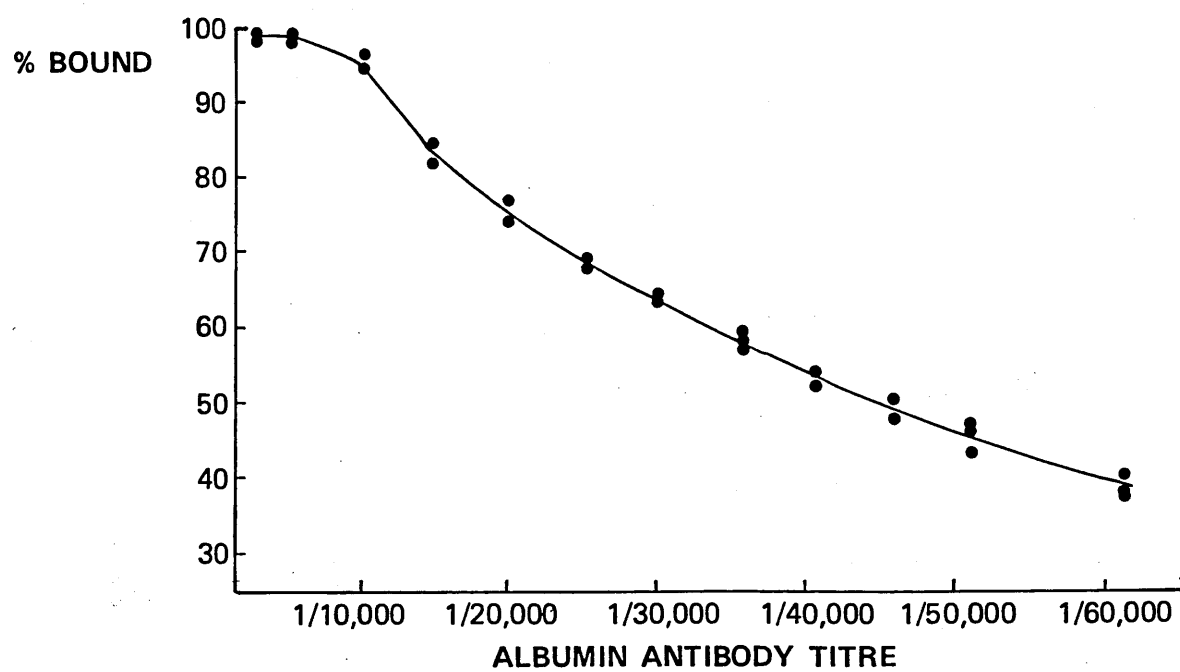


Figure 3. Albumin radioimmunoassay: Antibody binding curve. The titre 1:32,000 binds approximately 60% label.

d) Validation

Bovine serum albumin diluted in assay buffer to concentrations 0.1 - 10 µg/ml was assayed to check for cross-reaction. Figure 4 demonstrates that there is no change in the amount of label bound with increasing concentrations of bovine serum albumin, over the concentration range 0-10 µg/ml, showing that there was no cross-reaction of bovine albumin with antibody to human albumin. Human albumin, concentration 40 g/l, was glycated by incubation at 37°C for 1 week in 3 mM NaN₃ with and without 100 mmol/l glucose, then diluted in PbB to concentrations 0.1 - 10 µg/ml before assay. Figure 5 shows parallel standard curves, showing that native and glycated albumin reacted similarly with the antibody. A urine sample of albumin concentration 21.0 µg/ml was diluted serially before assay to check for parallelism (Figure 6). Standard curves and quality control samples were also assayed in PbB buffer containing glucose 27.8 mmol/l (5 g/100 ml). Figure 7 demonstrates that standard curves in PbB buffer with and without glucose run in parallel, showing that glucose has no effect on the binding of antibody.

e) Recoveries

Measured amounts of standard albumin solution were added to urine of known albumin concentrations and the solutions assayed to allow determination of recoveries. The mean recovery was 96.8% (range 94.0 - 98.2%; Table 2).

f) Quality control

All standard, quality control and unknown samples were assayed in triplicate. Standard curves were run at the beginning and end of each assay to check for assay drift. Four quality control samples were prepared from pooled urine samples, aliquoted and stored at -40°C. The concentration range of the control samples was 2 - 156 µg/ml. They were assayed on four occasions spread throughout each assay to allow calculation of intra- and inter-assay coefficients of variation $\frac{(SD \times 100)}{\text{mean}}$.

For each assay, the mean of each quality control sample and its coefficient of variation (CV) was calculated. After the first 10 assays, the overall mean and two standard deviations of each control sample were calculated. Subsequent assays were rejected if the intra-assay coefficient of variation for any quality control sample was >10%, or if any mean value was outwith the reference mean \pm 2SD limits or if the non-specific

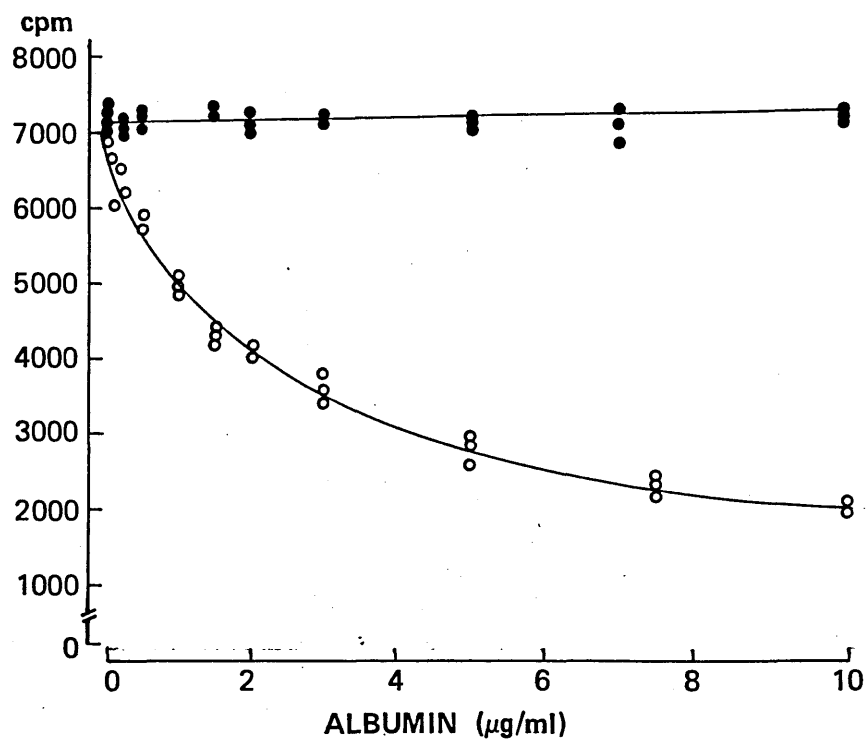


Figure 4. Albumin radioimmunosassay: Lack of binding of bovine serum albumin (0.1-10ug/ml) to anti-human albumin immunoglobulin.

Bovine albumin (●)

Human albumin (○)

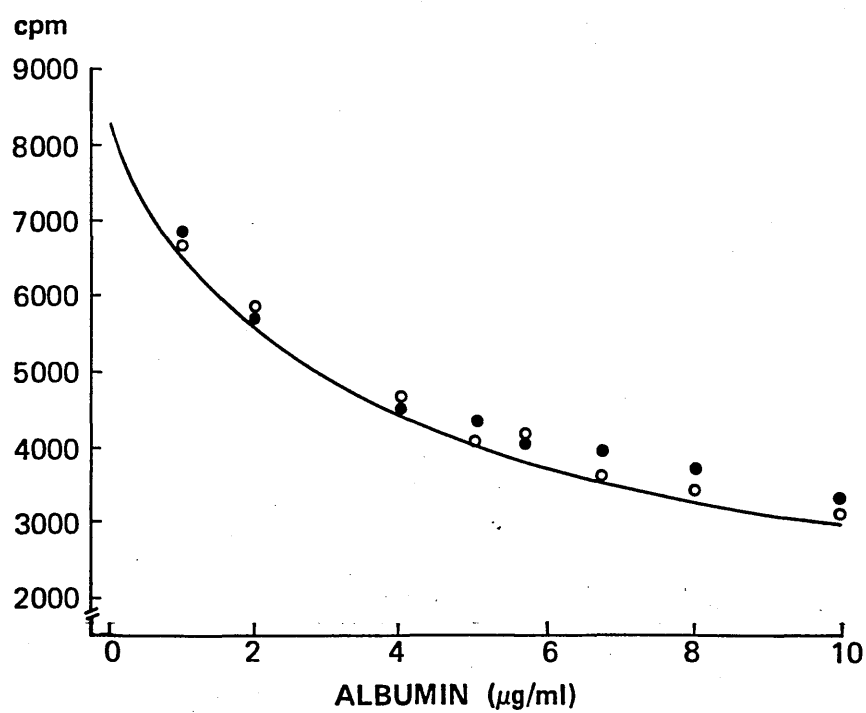


Figure 5. Albumin radioimmunoassay: Binding of native (o) and glycated albumin (●) to anti-human albumin immunoglobulin

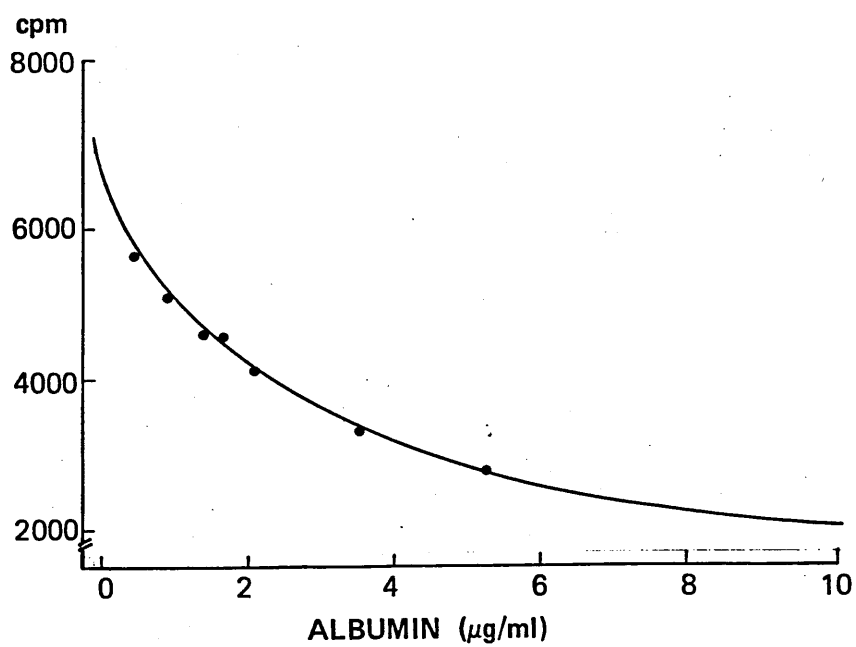


Figure 6. Albumin radioimmunoassay: Serial dilution of urine sample (●) containing 21.0 $\mu\text{g/ml}$ albumin, showing parallelism to standard curve.

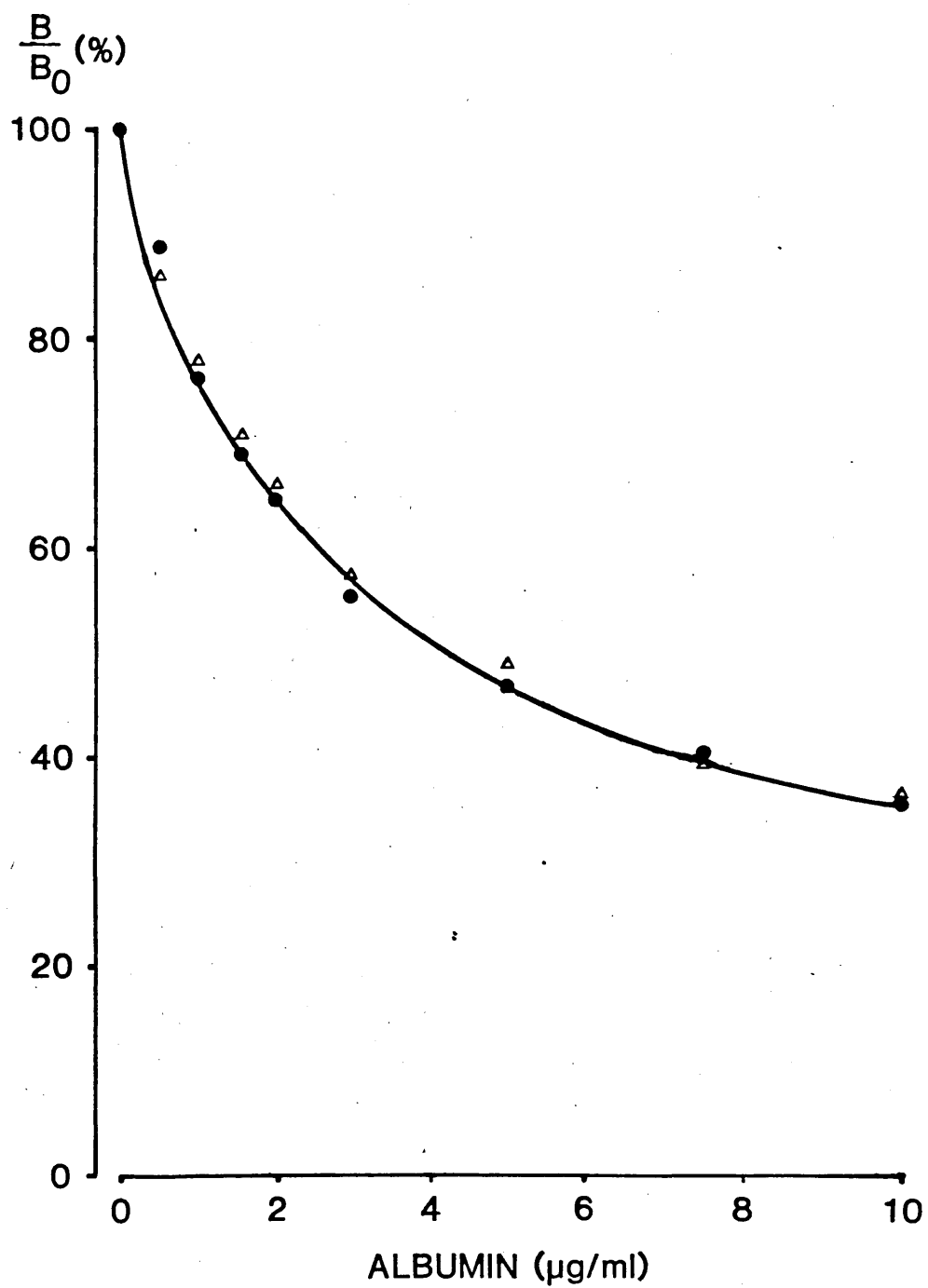


Figure 7. Albumin radioimmunoassay: Effect of glucose on antigen-antibody binding. Albumin standards prepared in buffer with (Δ) and without (\bullet) 27.8 mmol/l glucose.

Albumin Concentration ($\mu\text{g/ml}$)		Recovery (%)
Calculated	Measured	
10.0	9.7	97.0
8.0	7.8	97.5
6.7	6.5	97.0
5.7	5.6	98.2
5.0	4.9	98.0
4.0	3.9	97.5
2.0	1.9	95.0
1.0	0.94	94.0

Table 2 Albumin radioimmunoassay: Recoveries of known amounts of albumin added to urine.

binding exceeded 10% or the excess binding was <90%. Charts of the mean albumin concentration in each quality control sample in a series of assays are shown in **Figures 8 and 9**.

Representative intra- and inter-assay coefficients of variation of the controls are shown in **Table 3**.

g) Comparison with other sensitive albumin assays

The radioimmunoassay was compared with another albumin radioimmunoassay performed in the Department of Metabolic Medicine, Guy's Hospital Medical School, London(39). This radioimmunoassay uses rabbit antibody against human albumin and polyethylene glycol separation aided by the addition of human immunoglobulins. Twenty samples were run in each assay and one communal quality control sample has been measured in every assay in each laboratory for 4 years (QC3). The comparison of the 2 radioimmunoassays is shown in **Figure 10**. The correlation coefficient was 0.96 and the regression equation $y = 1.16x + 0.18$.

Over 4 years, the mean value for the quality control sample in the Newcastle assay was 20.3 µg/ml with a coefficient of variation of 3.9% and 21.0 µg/ml, with CV 4.1% in the Guy's Hospital assay.

The assay was also compared with an immunoturbidimetric assay developed by Dr P Bennett, Phoenix, Arizona, USA. Fifty samples were measured blindly in each assay. The correlation coefficient was 0.99 and regression equation $y = 1.02x - 0.30$ (**Figure 11**).

2.2 β₂-Microglobulin Assay

β₂-microglobulin was assayed by a modification of the method of Plesner et al(84).

a) Iodination

The iodination was carried out in 0.05M sodium dihydrogen phosphate buffer (Na.H₂.PO₄.H₂O), pH 7.5. Human β₂-microglobulin (Serotec Limited, Blackthorn, Bicester, Oxon, UK), 40 µg in 10 µl was added to a vial containing 2 mCi ¹²⁵I. Chloramine T, 10 µg in 10 µl was added and after mixing gently for 30 s, the reaction was terminated by the addition of 1% bovine serum albumin, 300 µl. The reaction

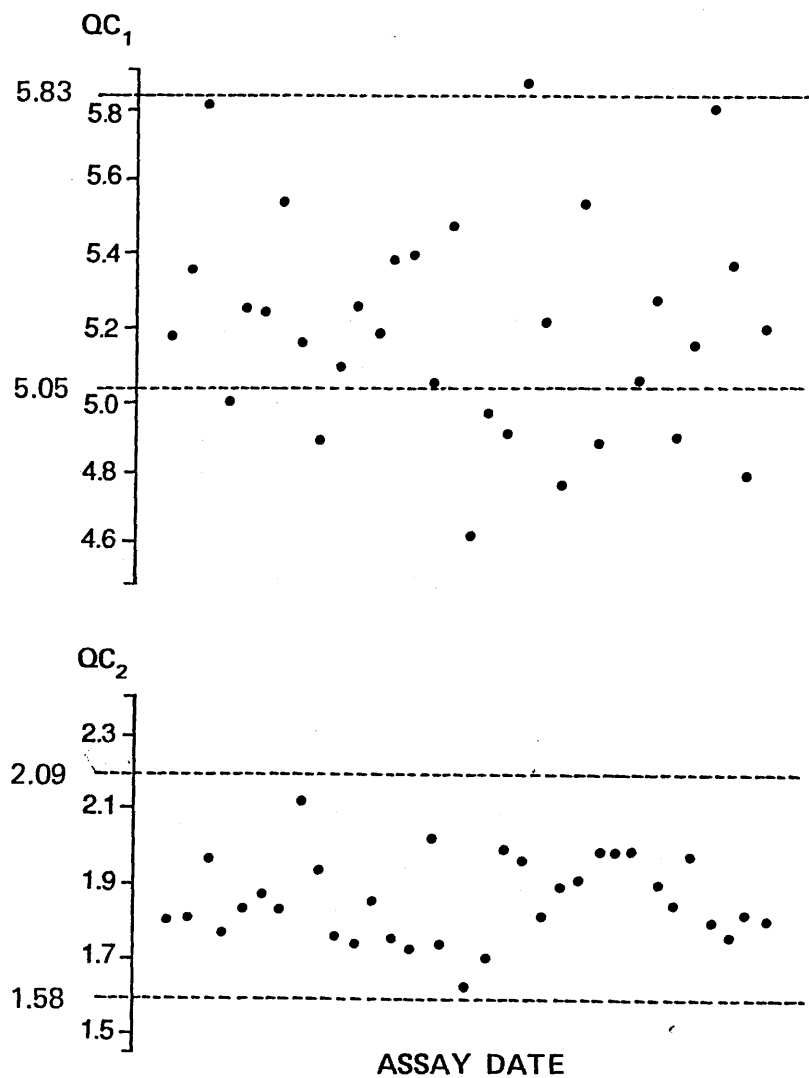


Figure 8 Albumin radioimmunoassay: Quality control charts; Variation of samples QC₁ (upper panel) and QC₂ (lower panel).

--- ± 2 SD of mean value of the sample in the first 10 assays.

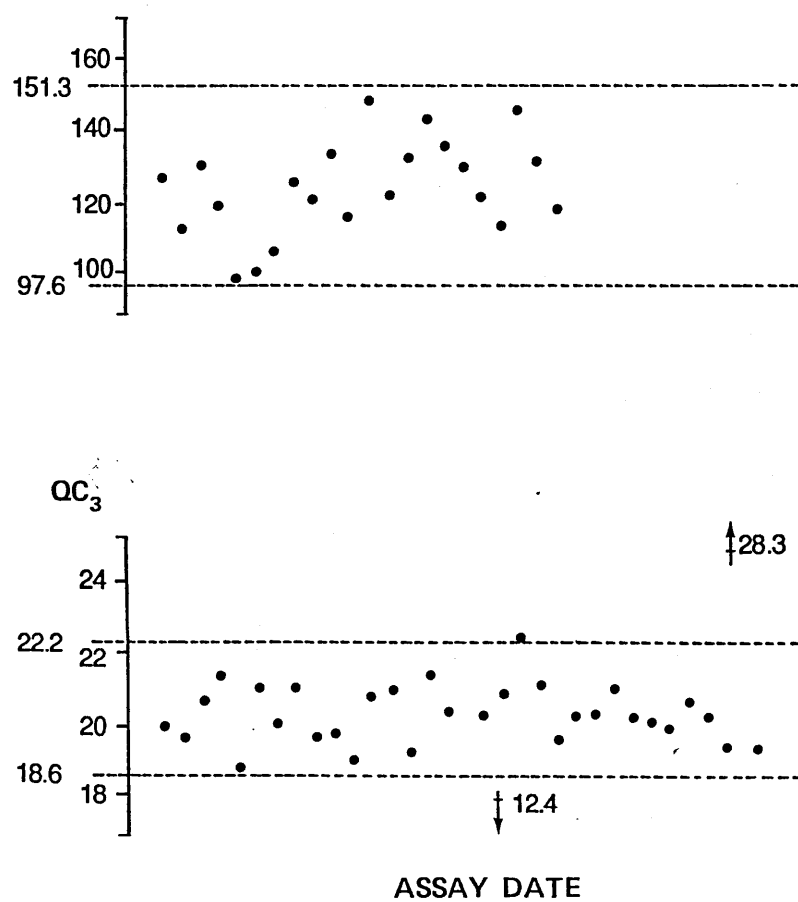


Figure 9 Albumin radioimmunoassay: Quality control charts; Variation of samples QC₄ (upper panel) and QC₃ (lower panel).

--- ± 2 SD of mean value of the sample in the first 10 assays.

Sample	Mean Concentration ($\mu\text{g/ml}$)	Coefficient of Variation (%)	
		Intra-assay	Inter-assay
QC ₁	5.2	2.3	5.7
QC ₂	1.8	4.2	7.0
QC ₃	20.4	3.8	4.4
QC ₄	124.4	6.5	10.8

Table 3 Albumin radioimmunoassay: Coefficients of variation of the quality control samples.

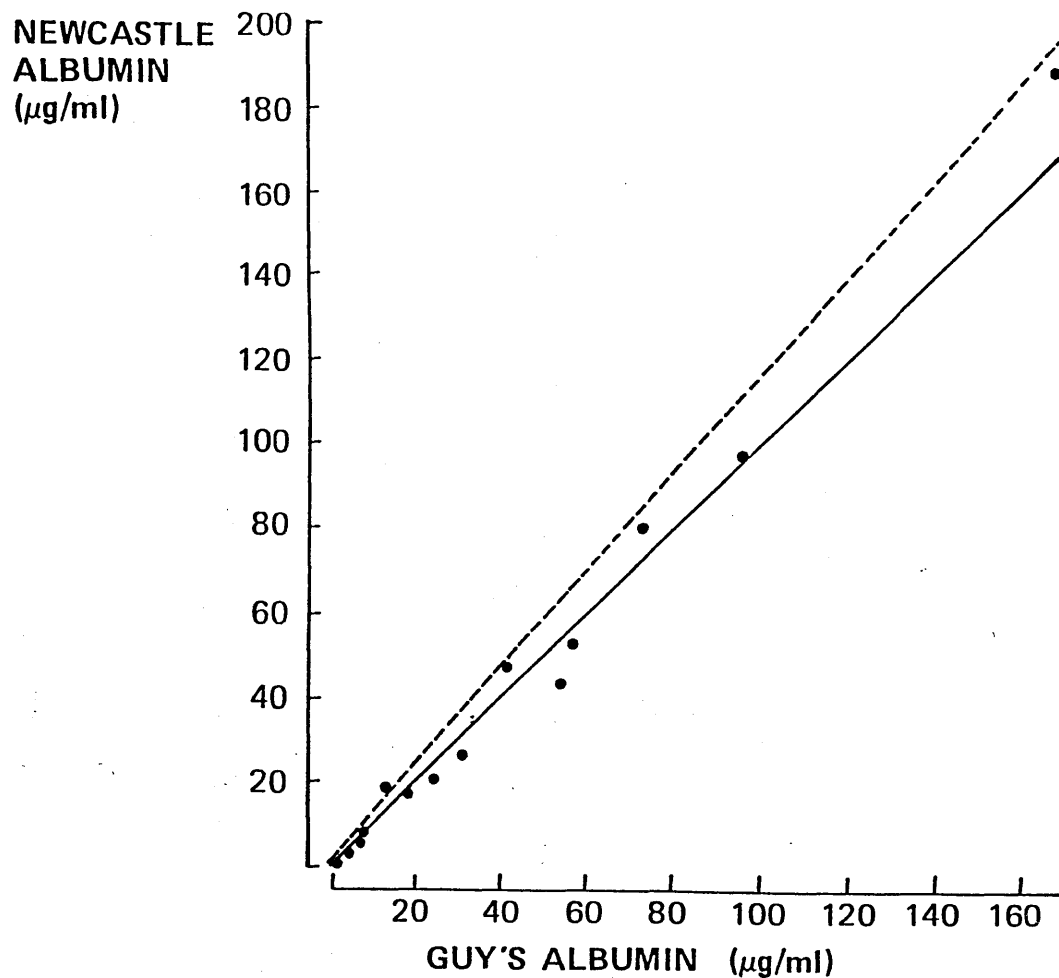


Figure 10 Albumin radioimmunoassay: Comparison of the albumin concentrations of 20 samples measured in the Newcastle (y-axis) and Guy's Hospital (x-axis) assays.

———— line of identity

- - - - calculated regression line, $y=1.16x+0.18$

$r=0.96$

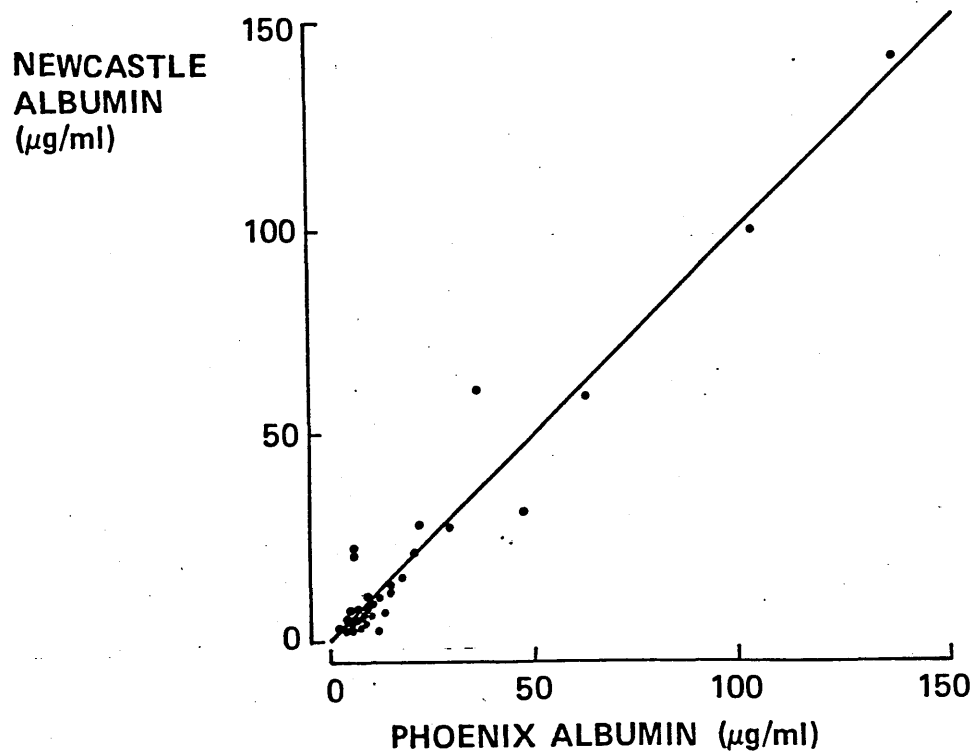


Figure 11 Albumin radioimmunoassay: Comparison of the albumin concentrations in 50 samples measured in the Newcastle radioimmunoassay (y-axis) and the Phoenix immunoturbidimetric assay (x-axis)

_____ line of identity

----- calculated regression line, $y=1.02x-0.30$

$r=0.99$

mixture was applied to a 1 by 50 cm column containing Ultragel AcA 54 (LKB, Bromma, Sweden). Elution was by 0.04M sodium dihydrogen phosphate buffer, pH 8.0, containing 0.2% (w/v) bovine serum albumin (PbB). Approximately 2 ml fractions were collected and 10 μ l aliquots counted for 30 s on an NE1600 multi-well gamma counter. The β_2 -microglobulin peak was pooled and stored at 4°C for use in the radioimmunoassay. Specific activity of the labelled protein and the percent ^{125}I bound were calculated as for the albumin assay. A typical elution profile is shown in Figure 12. The peak at fraction 44 is free ^{125}I , that at 17 ^{125}I albumin and at 29 ^{125}I β_2 -microglobulin. Fractions 29 and 30 were pooled to form the stock labelled β_2 -microglobulin solution. The specific activity was 47.5 $\mu\text{Ci}/\mu\text{g}$ and the percent ^{125}I bound 95.0.

b) β_2 Microglobulin Assay Protocol

β_2 -microglobulin standards were prepared from human β_2 -microglobulin in concentrations of 0 - 100 $\mu\text{g}/\text{l}$ in assay buffer PbB. Stock labelled β_2 -microglobulin was diluted 10 μ l in 6 ml PbB. Rabbit anti-human β_2 -microglobulin immunoglobulin (Dako, Copenhagen, Denmark) was diluted in PbB to a predetermined titre (see binding curve). To 100 μ l buffer, standard or unknown sample was added 100 μ l diluted label and 100 μ l diluted antibody. After mixing, the reaction was incubated at 4°C for 18 hours. Precipitation was by the addition of 500 μ l polyethylene glycol 6000, 25% (w/w) in assay buffer and 100 μ l donor calf bovine serum. The tubes were mixed and then centrifuged at 2400 rpm at 4°C for 50 min. The supernatant was decanted and the precipitate counted on a Packard Auto-Gamma 5780 counter. Total counts in each reaction tube were estimated by counting 100 μ l diluted label only. Non-specific binding was assessed by counting the precipitated radioactivity in a tube containing 100 μ l PbB buffer instead of diluted antibody. The binding of label to antibody was assessed by inclusion of "excess" reaction tubes containing 100 μ l antibody of titre 1:200 instead of diluted antibody. Non-specific binding was generally <10% and excess binding 90-95%. Serum samples were diluted 1:100 in PbB before assay. A typical standard curve is shown in Figure 13. The sensitivity of the assay was 2.5 $\mu\text{g}/\text{l}$.

c) Antibody Binding Curve

An antibody binding curve was performed for each new batch of label or antibody, with antibody diluted over a range of titres from 1:200 to 1:50,000. To 100 μ l assay buffer PbB was added 100 μ l diluted iodinated β_2 -microglobulin and 100 μ l diluted

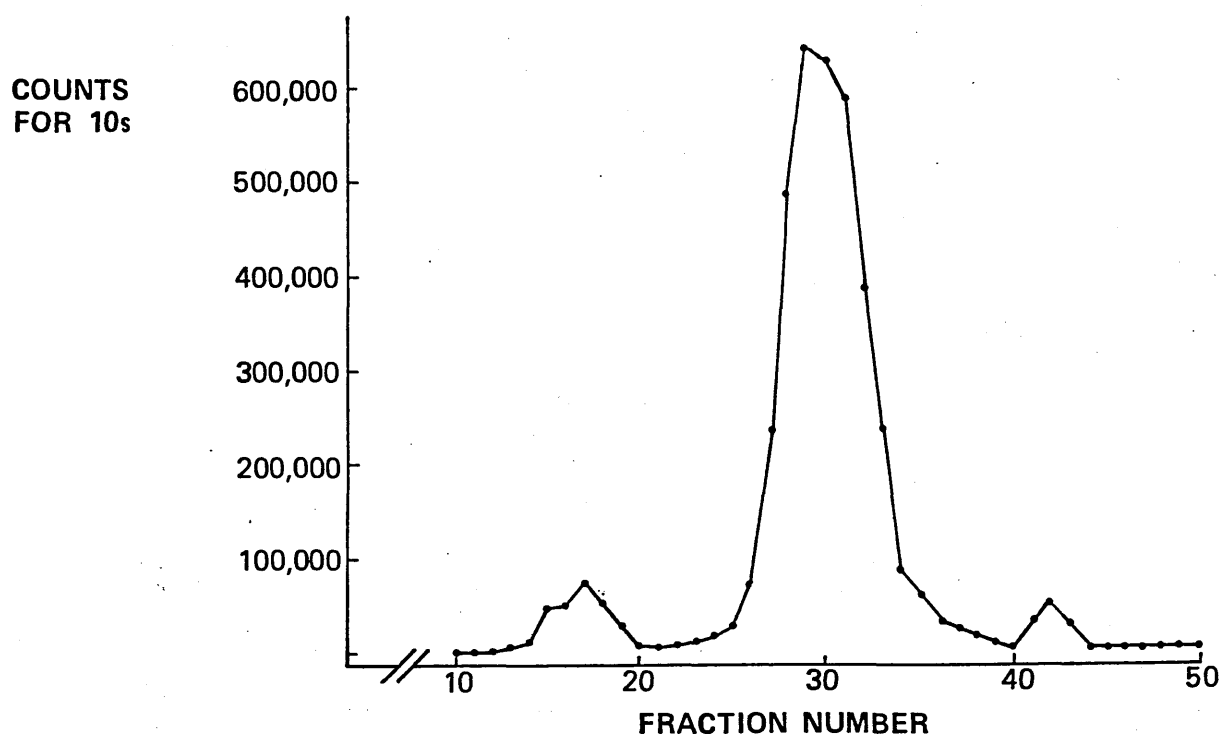


Figure 12 β_2 -Microglobulin radioimmunoassay: Iodination.
 Separation of ^{125}I β_2 -Microglobulin (peak at fraction 29) from ^{125}I albumin (peak at fraction 17) and free ^{125}I (peak at fraction 44).

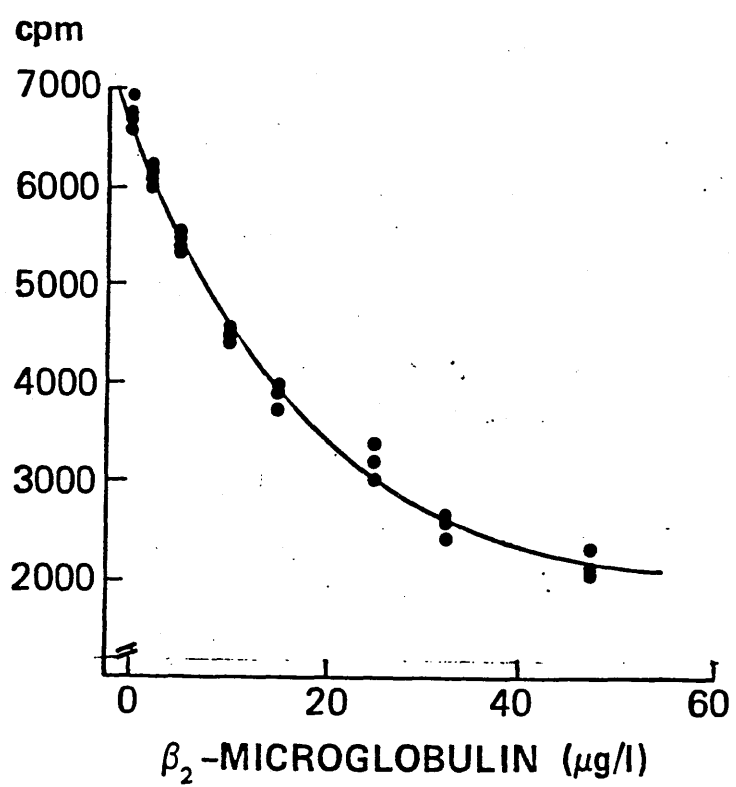


Figure 13 β_2 -Microglobulin radioimmunoassay: Standard curve.

antibody. The reaction was completed as described above. A typical curve is shown in Figure 14. The titre antibody used in subsequent assays was chosen to give binding of approximately 60%, usually 1:40,000.

d) Validation

Urine and serum samples were diluted serially before assay to check for parallelism. Figure 15 shows that serial dilutions of urine and serum samples ran parallel to the standard curve.

e) Recoveries

To each of 3 urine and 3 serum samples containing a predetermined concentration of β_2 -microglobulin was added a known amount of human β_2 -microglobulin standard prior to reassay. Recoveries are shown in Table 4. The mean recovery for urine was 93.5 (85.1 - 102.9)% and for serum 99.1 (90.0 - 111.5)%.

f) Quality Control

All standard, quality control and unknown samples were assayed in triplicate. Standard curves were run at the beginning and end of each assay. Four urine samples, concentration range 4.0 - 36.0 $\mu\text{g/l}$ and three serum samples, range 1.3 - 1.8 mg/l were assayed on four occasions in each assay to check precision. Intra- and inter-assay coefficients of variation were calculated (Tables 5 and 6) and the same criteria for assay acceptance used as in the albumin assay.

2.3. Urine Samples

Timed overnight urine collections were made into 0.5ml 0.1% thiomersal (Sigma Chemical Company, Poole, Dorset, UK) and 0.5 ml 4M NaOH (Sigma Chemical Company, Poole, Dorset, UK). Patients were instructed to empty their bladder, discarding the urine, immediately before retiring to bed. The time was noted on the container. All urine passed during the night was collected. Immediately on rising, the bladder was emptied and all the urine collected into the container. The time was again noted. From the volume of the sample (ml) and the duration of the collection (min) the albumin excretion rate (AER) was calculated:

$$\text{AER} = \frac{\text{albumin concentration } (\mu\text{g/ml}) \times \text{volume (ml)}}{\text{duration (min)}} \quad \mu\text{g/min}$$

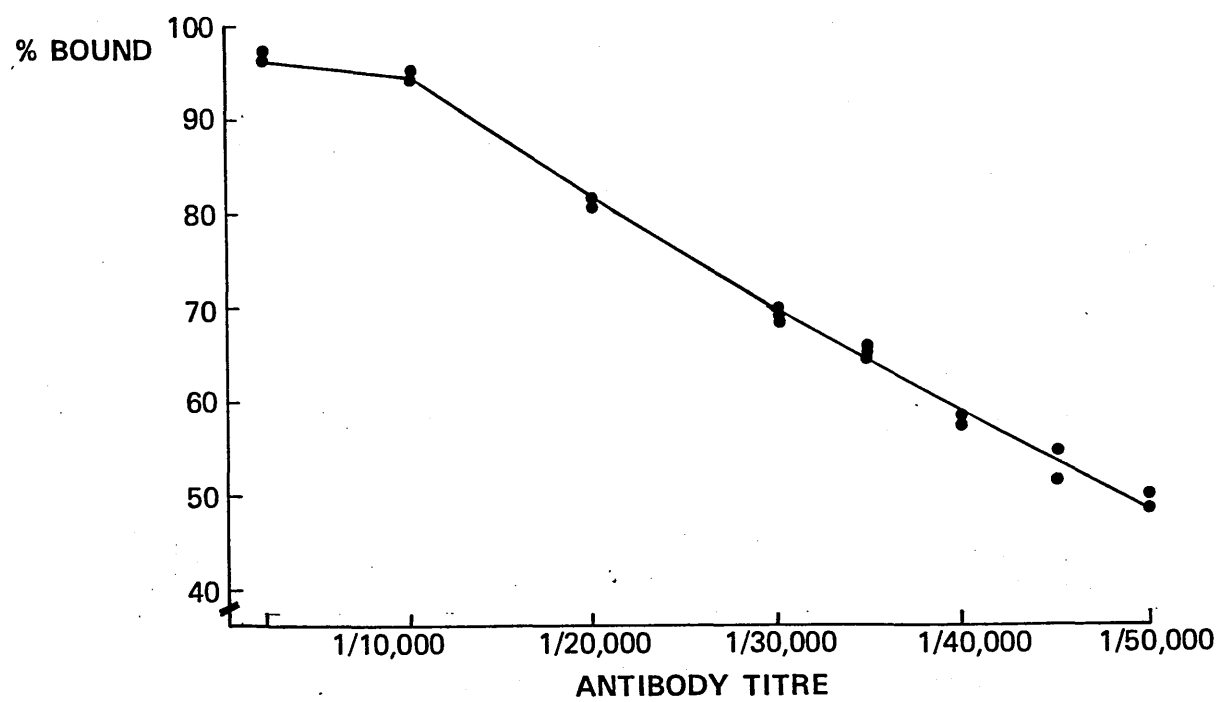


Figure 14 β_2 -Microglobulin radioimmunoassay: Antibody binding curve. A titre of 1:40,000 binds approximately 60% label.

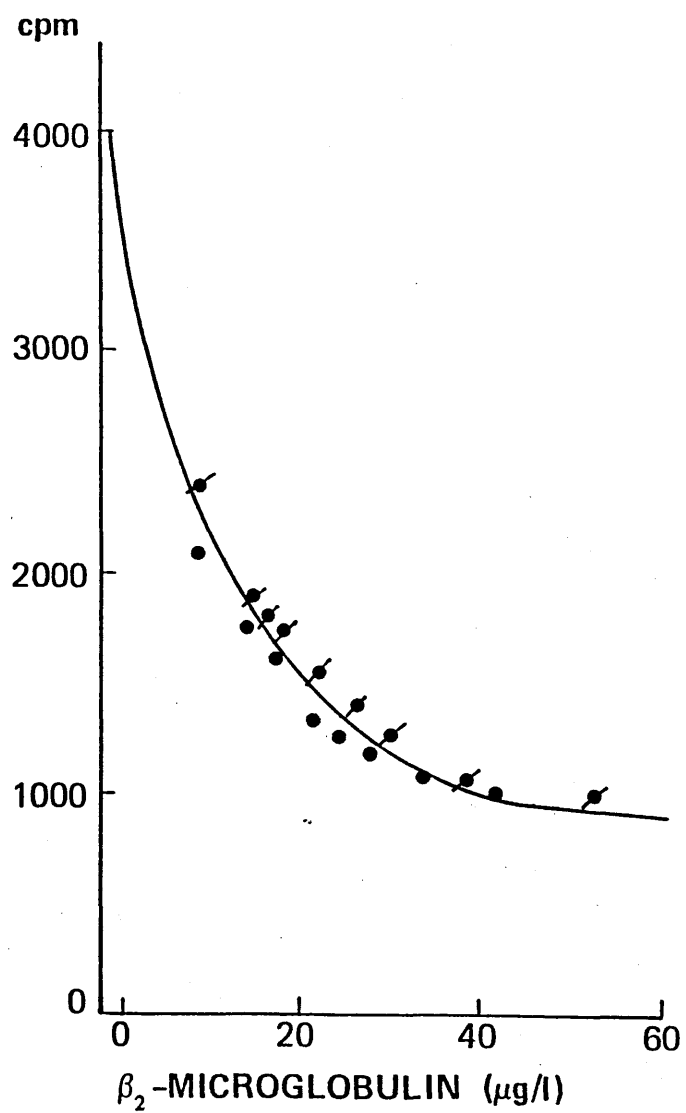


Figure 15 β_2 -Microglobulin radioimmunoassay: Serial dilution of urine (○) and serum samples (●), both showing parallelism to the β_2 -microglobulin standard curve.

Urine			Serum		
Concentration ($\mu\text{g/l}$)		Recovery (%)	Concentration ($\mu\text{g/l}$)		Recovery (%)
Measured	Calculated		Measured	Calculated	

8.0	9.4	85.1	15.5	15.6	99.4
13.5	14.0	96.4	17.6	16.0	90.9
15.4	16.5	93.3	19.0	20.1	94.5
19.6	21.5	91.2	21.9	21.8	100.5
35.0	34.0	102.9	26.2	23.5	111.5
36.0	39.0	92.3	33.0	33.8	97.6

Table 4 β_2 -Microglobulin radioimmunoassay: Recoveries of β_2 -microglobulin in urine and serum

Sample	Mean Concentration ($\mu\text{g/l}$)	Coefficient of Variation (%)	
		Intra-assay	Inter-assay
1	6.4	7.2	7.7
2	33.5	4.0	6.1
3	39.5	6.0	3.8

Table 5 β_2 -Microglobulin radioimmunoassay: Urine Quality Control Sample

Sample	Mean Concentration (mg/l)	Coefficient of Variation (%)	
		Intra-assay	Inter-assay
1	1.45	2.8	6.0
2	1.65	6.0	6.0
3	1.70	5.4	3.4

Table 6 β_2 -Microglobulin radioimmunoassay: Serum Quality Control Samples

Aliquots of the first morning urine passed immediately on rising (EMU) were collected with no preservative. Aliquots of urine were stored at -40°C until assay. All samples were centrifuged at 2400 rpm at 4°C for 10 min immediately before assay. Every urine sample was screened for infection using Nephurtest strips (Boehringer, Mannheim, West Germany). Any sample giving a positive leucocyte or nitrite reaction was discarded, to avoid falsely high values due to inflammation of the urinary tract.

2.4. Blood Measurements

Serum for β_2 -microglobulin measurement was stored at -40°C before assay. Whole blood for determination of glycosylated haemoglobin was collected into EDTA before assay by the Corning electrophoresis method. The coefficient of variation is 8.0% at a glycosylated haemoglobin concentration of 10% and the reference range 5.0-7.5%. Plasma and urine creatinine were determined by a modified Jaffe reaction (85) using a Beckman Astra automated multi-channel analyser. Serum for fructosamine determination was stored at -40°C before assay by a colourimetric technique using nitroblue tetrazolium (86). The reference range was 1.79 - 2.35 mmol fructosamine/100g albumin.

2.5. Assay of urinary human epidermal growth factor

Human epidermal growth factor (hEGF) was measured in triplicate by a double antibody radioimmunoassay(87). hEGF, kindly donated by Dr Gregory, Imperial Chemical Industries Ltd, Macclesfield, Cheshire, UK, was iodinated to specific activities of 90 - 100 Ci/g by the iodogen method(88). To either 0.1 ml purified hEGF standard or urine diluted five-fold was added 0.1 ml specific rabbit anti-hEGF serum(89) at a final dilution of 1:24,000 and 0.1 ml ^{125}I -EGF (10,000 cpm). The antiserum used bound 10 - 15% of total radioactivity added in the absence of unlabelled hEGF. After incubation at 4°C for 24 h, the bound and free fractions were separated by addition of a second antibody linked to cellulose (Sac-Cel, Wellcome Reagents Ltd, Beckenham, Kent, England). The radioactivity of the precipitate was counted in a multi-well gamma counter (Gambyt CS-20) and results calculated using a standard logistic curve-fit. All samples were assayed in the same batch, the intra-assay coefficient of variation being 2.6%. Figure 16 shows a representative dose-response curve for purified hEGF standard compared to that given by dilutions of urine in the homologous hEGF assay.

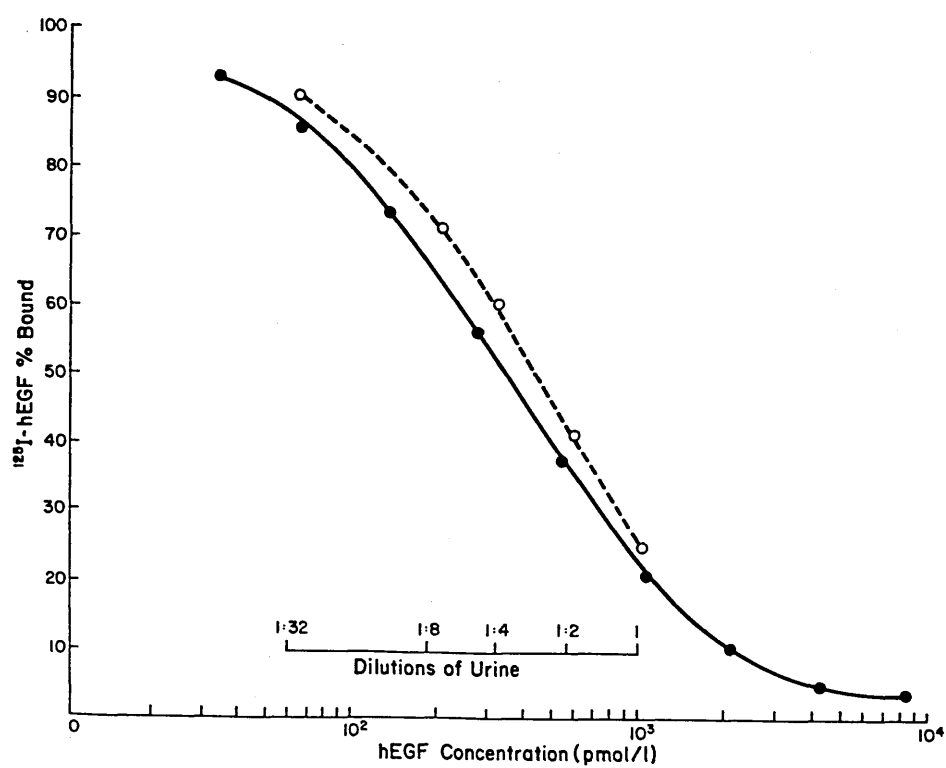


Figure 16 Epidermal growth factor radioimmunoassay: Dose response curves for purified hEGF standard (●) and dilutions of urine (○). Each point represents the mean of 3 determinations.

2.6 Ethical Approval

Where appropriate, studies were approved by the Newcastle Area Health Authorities Joint Ethical Committee.

CHAPTER 3

3. Albumin excretion in healthy adults and children

3.1 Aims

The aims of this study were to determine normal ranges for albumin excretion in healthy adults and children. Given the difficulties of collecting complete urine samples from young children, albumin excretion has also been expressed as albumin:creatinine ratio.

3.2 Methods

Timed overnight urine samples were collected from 106 adults and 64 children. All were healthy and taking no medication. The age and sex of each subject were recorded. Albumin was measured in all samples for calculation of the albumin excretion rate and in children's samples, creatinine was also estimated, to allow determination of the albumin:creatinine ratio. Statistical analysis was by Student's t test and Pearson correlation coefficient, as appropriate, in the adult population and by non-parametric means in the children.

3.3 Results

(a) Albumin excretion in normal adult subjects

Details of the characteristics of the normal subjects and AER's are shown in Table 7. Males were older than females ($p < 0.001$) and had a significantly higher AER ($p < 0.005$). The distribution of the AER in the population was not normal (Figure 17). Logarithmic transformation to base 10 resulted in a normally distributed population and hence logarithmic transformation of AER has been done before statistical analysis in all further adult studies. AER results are therefore given as geometric mean $\bar{x} \div 2$ tolerance factors.

There was no correlation of AER with age in the whole group or in either sex.

(b) Albumin excretion in healthy children

After logarithmic transformation, the AER in the 64 normal children was 2.5(0.6–7.0) $\mu\text{g}/\text{min}$ (geometric mean $\bar{x} \div 2$ tolerance factors), significantly lower than in the adult population [5.4(1.7–10.5) $\mu\text{g}/\text{min}$; $p < 0.001$]. However, albumin excretion rates and albumin:creatinine ratios were not normally distributed even after logarithmic transformation in the children. The results have therefore been analysed using non-

parametric tests: the Kendall Rank and Partial Rank correlations and Mann-Whitney U test. Details of the children's results so analysed are given in Table 8. Excretion rates were similar in boys and girls but there was a strong correlation of AER with age ($\rho = 0.33$, $p < 0.001$; Figure 18). Albumin:creatinine ratios were similar in boys and girls, with no correlation with age.

3.4 Discussion

It is difficult to compare the normal ranges established here with others because of differences in urine collections and different methods for albumin determination. In the only large published study of albumin excretion rate, measured by radioimmunoassay, in a timed overnight urine sample, the geometric mean (95% tolerance limits) for AER was 3.2 (1.2-8.6) $\mu\text{g}/\text{min}$ (90). In this study, the distribution of AER was also positively skewed, but normalised by logarithmic transformation. No relationship of AER to age or sex was seen. In other studies on small numbers of healthy adults, the AER has been reported as 3.7 ± 2.1 (91), 0.5-10.4(92), 4.5 ± 1.5 (37), <20 (47) and <40 $\mu\text{g}/\text{min}$ (46). It would thus seem that the normal range found here is not unrealistic.

Similarly, the median overnight excretion rate of 2.5 $\mu\text{g}/\text{min}$ in normal children compares well with that of Davies(58) but not of Rowe(53) who used an immunoturbidimetric technique for albumin determination. The latter is known to give consistently lower results than values measured by radioimmunoassay(93). Davies also found a correlation between age and day and night albumin excretion rates in a large group of 374 normal children(58) whereas 2 smaller studies did not(53,56). The reasons for these differences are unclear.

The demonstration that albumin excretion rates are significantly lower in children than adults, and the relationship of AER to age in children, has important implications for studies examining albumin excretion in diabetic children. Age-matched controls will obviously be required. The adult discriminant AER values for predicting increased risk are not applicable to children.

	All	Male	Female
Number	106	68	38
Age (years) ¹	43±18 (17-80)	47±19** (17-80)	36±13 (22-69)
AER (µg/min) ²	5.4 (1.7-10.5)	5.9 (2.0-11.2)*	4.4 (1.4-9.3)

¹ mean±2SD (range)

² geometric mean (x/÷2 tolerance factors)

*, **: p<0.005, p<0.001, males versus females

Table 7 Albumin excretion rates (AER) in the normal adult population.

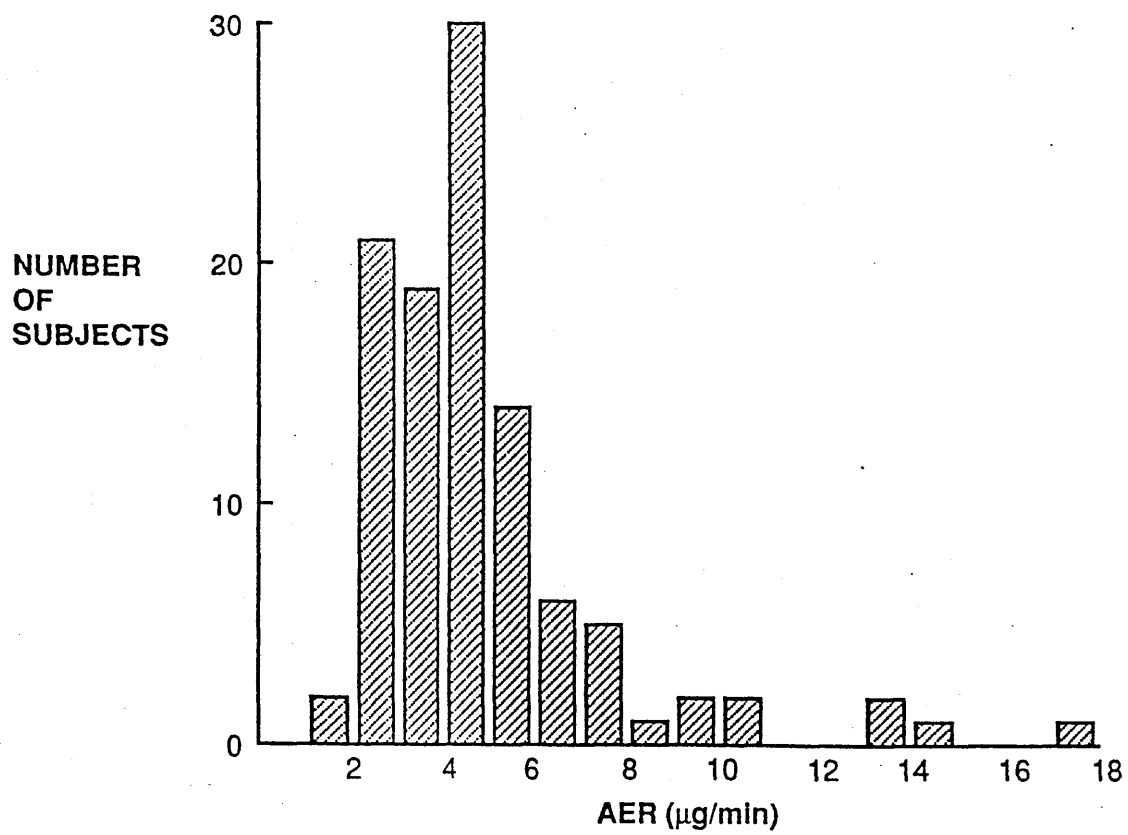


Figure 17 Distribution of albumin excretion rates (AER) in the normal adult population.

	All	Male	Female
Number	64	32	32
Age ¹ (years)	9.0 (3-17)	8.0 (3-17)	9.9 (4-15)
AER ² (µg/min)	2.4 (0.2-20.8)	2.2 (0.2-20.8)	2.4 (0.2-10.6)
Albumin: creatinine ratio ² (µg/mmol)	0.95 (0.40-3.7)	0.84 (0.4-5.85)	0.96 (0.43-7.21)

1 mean (range)

2 median (range)

Table 8 Albumin excretion in healthy children.

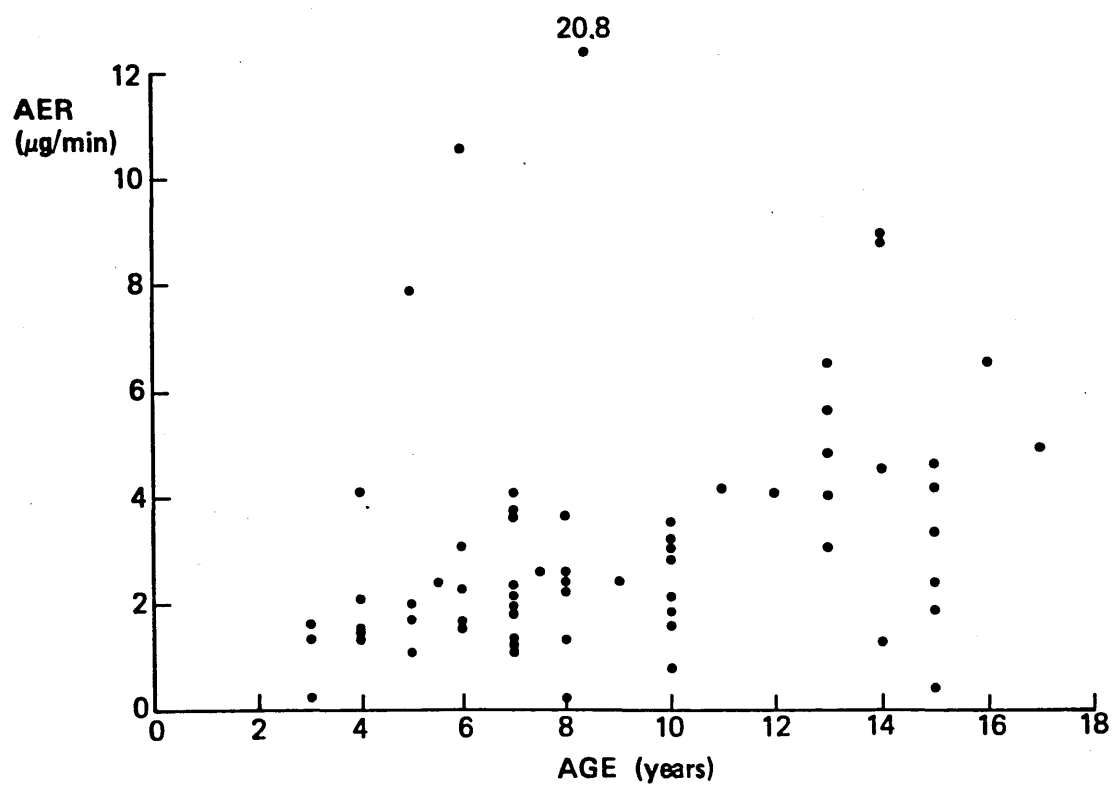


Figure 18 Albumin excretion in healthy children: Effect of age

$r = 0.33$

$p < 0.001$

CHAPTER 4

Comparison of the prevalence and associated features of abnormal albumin excretion in insulin-dependent and non-insulin dependent diabetes

4.1 Aims

The aims of this study were to establish the prevalence of the various degrees of abnormal albumin excretion in an unselected diabetic out-patient population and to compare insulin-dependent and non-insulin dependent diabetic patients. The relationship of albumin excretion to age, sex, duration of diabetes, blood pressure, glycaemic control and the presence of the other complications of diabetes is examined.

4.2 Subjects and Method

Diabetic patients

All adult diabetic patients attending 3 hospital diabetic clinics over a 6 month period were asked to provide a timed overnight urine sample. Written instructions and containers were distributed at one clinic visit and the patients asked to collect the sample on the night before their next clinic visit. The prevalence of the various degrees of albuminuria in the clinic population was determined. Patients were classified as insulin-dependent if they had commenced on insulin therapy within one month of diagnosis and remained on it continuously since. Those not fulfilling these criteria were classified as non-insulin dependent. Four patients with secondary diabetes were excluded. No patients were studied within 6 months of diagnosis of diabetes or within 1 month of acute metabolic decompensation.

Clinical data

For each diabetic patient who provided a timed overnight urine sample as above, information about the age, sex, known duration and treatment of diabetes (diet alone, diet plus oral hypoglycaemic agents, diet plus insulin) was obtained from the medical records. Non-insulin dependent patients were subdivided into three groups on the basis of treatment with diet alone, oral hypoglycaemic agents or insulin. Treatment of hypertension and any other medical condition was noted, as was the presence or absence of the other complications of diabetes. Retinopathy was assessed by fundoscopy through dilated pupils. Retinopathy was defined as any noted abnormality typical of diabetes; peripheral vascular disease as symptomatic intermittent claudication or absent dorsalis pedis and posterior tibial pulses or a past or present history of gangrene or amputation; peripheral neuropathy as symptoms of paraesthesiae or typical stocking sensory deficit and absent ankle jerks; ischaemic heart disease as a

typical history of angina pectoris or a previous myocardial infarction as judged by history, electro-cardiographic and enzymatic changes. A history of non-diabetic renal disease was also noted. Blood pressure was measured on the day of the clinic visit (sitting, after 5 min rest, to the nearest 2mm Hg, diastolic phase V, using a standard cuff) and blood drawn for glycosylated haemoglobin estimation.

Statistical methods

All statistical analysis was carried out using the Statistical Package for the Social Services (94). Albumin excretion rates in the normal and diabetic populations were positively skewed, hence the rates have been logarithmically transformed before analysis. Differences between groups were assessed by Student's t test or χ^2 test as appropriate. Individual correlations were assessed by the Pearson correlation coefficient and multiple relationships by multivariate analysis using step-wise entry, with log AER as the dependent variable.

4.3 Results

Prevalence of the various degrees of albuminuria

Of the 1456 subjects approached, 1031 (71%) provided a timed overnight urine sample. Seventy nine (75 women and 4 men) gave a positive Nephur test for blood, leucocytes or nitrites and were excluded. Twelve men had a history of other renal tract disease (six malignancies, three calculi, one glomerulonephritis, one renal tuberculosis, one congenital abnormality) and were also excluded, leaving 940 for analysis. Five hundred and twenty four were defined as NIDDM and 416 as IDDM. Of the 940 patients, 671 (71.4%) had AER within the normal range ($\leq 10.5 \mu\text{g}/\text{min}$), 138 (14.7%) had AER between 10.6 and 29.9 $\mu\text{g}/\text{min}$, (elevated excretion but not known to be at risk), 79 (8.4%) had AER 30-150 $\mu\text{g}/\text{min}$ (at risk microalbuminuria) and 52 (5.5%) AER $>150 \mu\text{g}/\text{min}$ (approaching clinical nephropathy). The corresponding figures for IDDM and NIDDM are given in Table 9. The mean log AER was higher in the NIDDM group ($t=2.62$, $p<0.009$) and the proportion of NIDDM patients with abnormal rates of albumin excretion was higher than in IDDM ($\chi^2=15.2$, $p<0.002$).

Association of albuminuria with other clinical features

Because of the difference in albumin excretion, patients with insulin-dependent and non-insulin dependent diabetes have been analysed separately. Details of the patient characteristics are given in Table 10. As might be expected, non-insulin dependent diabetic patients were older at the time of study than insulin-dependent patients, older

AER ($\mu\text{g}/\text{min}$)				
	≤ 10.5	10.6-29.9	30-150	>150
All patients	671 (71.4)	138 (14.7)	79 (8.4)	52 (5.5)
IDDM	323 (77.7)	45 (10.8)	28 (6.7)	20 (4.8)
NIDDM	348 (66.4)	93 (17.8)	51 (9.7)	32 (6.1)

Number (%)

Table 9 Distribution of the various levels of albumin excretion rate (AER) in the total diabetic population and IDDM and NIDDM patients.

	IDDM	NIDDM
Sex M:F	215:201	272:252
Age (years)	45±16 (14-87)	65±10*** (19-86)
Duration of diabetes (years)	18±12 (1-55)	8±7*** (1-33)
Age at diagnosis (years)	27±14 (1-71)	57±11*** (19-85)
Glycosylated haemoglobin (%)	10.0±2.1 (5.5-19.0)	10.1±2.7 (4.9-18.9)
Systolic BP (mmHg)	135±23	151±25***
Diastolic BP (mmHg)	77±10	83±11***

Mean ± SD (range)

*** p<0.001 IDDM versus NIDDM

Table 10 Characteristics of insulin-dependent and non-insulin dependent diabetic patients.

at diagnosis and had a shorter duration of diabetes. The prevalence of other long-term complications of diabetes and of treated hypertension is given in Table 11. Retinopathy was commoner in IDDM but peripheral vascular disease, ischaemic heart disease and treated hypertension were more frequent in NIDDM. Because of small numbers, all patients with retinopathy, whether background or proliferative, have been analysed together. performed on data from all patients,

Univariate analysis showed that in IDDM, AER was related to known duration of diabetes ($r=0.21$, $p<0.001$), systolic blood pressure ($r=0.18$, $p<0.001$), male sex ($t=2.46$, $p<0.002$), and the presence of retinopathy ($t=4.61$, $p<0.001$), peripheral vascular disease ($t=2.85$, $p<0.01$) and peripheral neuropathy ($t=2.50$, $p<0.02$). In NIDDM, AER correlated with known duration of diabetes, ($r=0.18$, $p<0.001$), systolic ($r=0.20$, $p<0.001$) and diastolic blood pressure ($r=0.17$, $p<0.001$) and to the presence of peripheral vascular disease ($t=4.12$, $p<0.001$), retinopathy ($t=3.66$, $p<0.001$) and ischaemic heart disease ($t=2.64$, $p<0.002$). Tables 12 and 13 demonstrate the effect of the various variables on the four categories of albuminuria. In IDDM, duration of diabetes was significantly increased in the three categories of abnormal excretion compared with the normoalbuminuric patients. Systolic blood pressure increased as AER increased, but reached significance only in the group with AER $>150 \mu\text{g}/\text{min}$. The prevalence of retinopathy was more than doubled in those with AER $>150 \mu\text{g}/\text{min}$ compared with the normoalbuminuric patients. In NIDDM, those patients with elevated AER had a longer duration of diabetes. Systolic blood pressure was significantly increased in all abnormal groups compared with the normoalbuminuric group, whereas diastolic BP was increased only in the range of AER $30\text{--}150 \mu\text{g}/\text{min}$. Retinopathy, peripheral vascular disease and ischaemic heart disease were commoner in those patients with abnormal albumin excretion.

Multiple regression analysis, using log AER as the dependent variable and with all factors individually correlating with log AER entered in step-wise fashion, revealed that in IDDM, only male sex and presence of retinopathy remained significant ($r=0.28$, $p<0.001$). A regression equation was formulated:

$$\begin{aligned} \log \text{AER} = & 0.705 + (0.243 \times \log \text{AER if retinopathy}) \\ & + (0.146 \times \log \text{AER if male}) \end{aligned}$$

Thus the ratio of AER with and without retinopathy is 1.75 and male to female is 1.40. Figure 19 illustrates the effect of sex and Figure 20 of retinopathy in AER in IDDM. At all levels of albumin excretion, AER is higher in males and in those with retinopathy.

	IDDM n (%)	NIDDM n (%)
Retinopathy	125 (30.1)	96 (18.3)***
Peripheral vascular disease	28 (6.8)	78 (14.9)***
Ischaemic heart disease	34 (8.2)	122 (23.4)***
Peripheral neuropathy	31 (7.5)	59 (11.3)
Treated hypertension	21 (5.0)	94 (18.0)***

*** p< 0.001

Table 11 Presence of other complications and of treated hypertension in the two groups of diabetic patients.

Factor	AER ($\mu\text{g}/\text{min}$)			
	≤ 10.5	10.6-29.9	30-150	>150
Duration (years)	17 \pm 11	22 \pm 12**	22 \pm 12*	25 \pm 12***
Systolic BP (mmHg)	133 \pm 22	137 \pm 23	140 \pm 23	150 \pm 22**
Male sex (%)	51	52	69	45
Retinopathy (%)	27	41**	41	69***

Mean \pm SD

*, **, *** $p < 0.05$, $p < 0.02$, $p < 0.005$ versus normoalbuminuric patients

Table 12 Effect of duration of diabetes, systolic blood pressure, male sex and the presence of retinopathy on the 4 ranges of albumin excretion in IDDM.

Factor	AER ($\mu\text{g}/\text{min}$)			
	≤ 10.5	10.6-29.9	30-150	>150
Duration (years)	7 \pm 7	9 \pm 8*	10 \pm 9*	10 \pm 7*
Systolic BP (mmHg)	147 \pm 24	157 \pm 27***	157 \pm 25**	162 \pm 26***
Diastolic BP (mmHg)	82 \pm 11	84 \pm 12	89 \pm 11***	85 \pm 10
Retinopathy (%)	15	22	35***	38**
PVD (%)	10	21***	29***	31***
Ischaemic heart disease (%)	19	29*	38***	35

Mean \pm SD

*, **, *** $p < 0.05$, $p < 0.02$, $p < 0.005$ versus normoalbuminuric patients

PVD: Peripheral vascular disease

Table 13 Effect of duration of diabetes, systolic and diastolic blood pressure and other complications on the four ranges of albumin excretion in NIDDM.

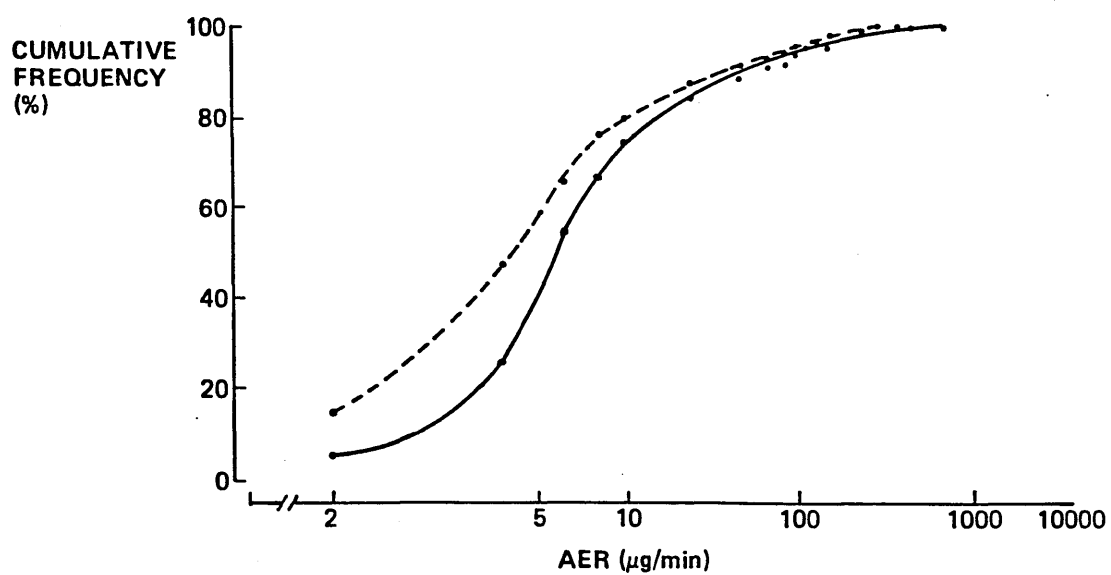


Figure 19 The influence of sex on albumin excretion rate (AER) in IDDM.

male —————
female - - - - -

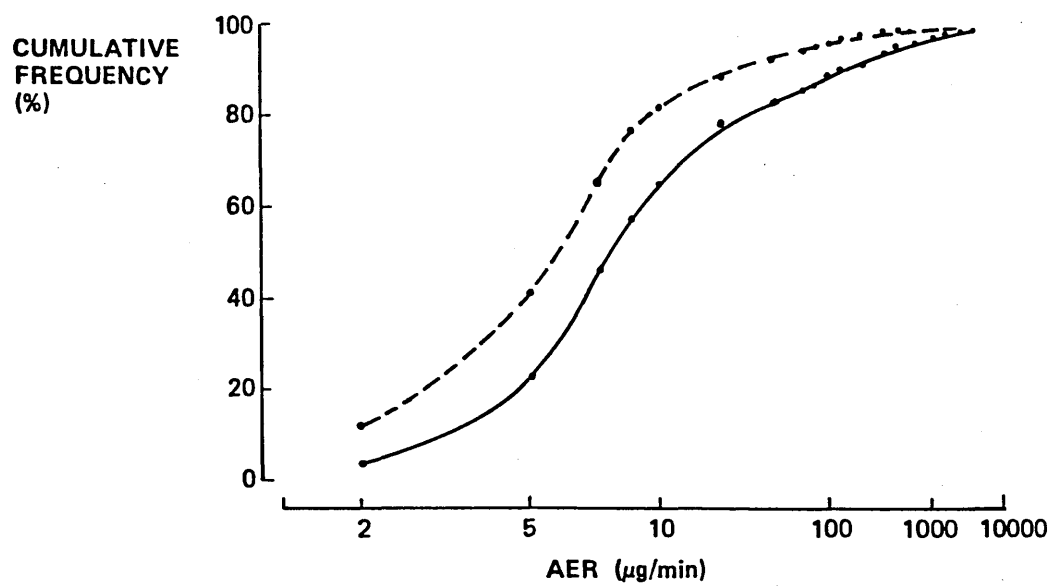


Figure 20 The influence of retinopathy on albumin excretion rate (AER) in IDDM.

retinopathy —————
 no retinopathy - - - - -

For NIDDM, multiple regression analysis showed a significant relationship of log AER to systolic blood pressure, peripheral vascular disease and retinopathy ($r=0.31$, $p<0.001$), such that:

$$\begin{aligned}\log AER = & 0.249 + (0.004 \text{ per mm SBP}) \\ & + (0.266 \times \log AER \text{ if PVD}) \\ & + (0.179 \times \log AER \text{ if retinopathy})\end{aligned}$$

Thus for each mm Hg systolic blood pressure, AER increases by a factor of 1.01 and the ratio of AER with and without retinopathy is 1.51 and with and without peripheral vascular disease is 1.85. Figures 21 and 22 show the effect of retinopathy and peripheral vascular disease on AER in NIDDM and Figure 23 the effect of systolic blood pressure. Again, the effect of all three variables is seen at all levels of AER.

At-risk microalbuminuria (AER 30-150 $\mu\text{g}/\text{min}$) was observed at all durations of diabetes in NIDDM patients. By contrast, in IDDM only one patient with duration of diabetes less than 5 years and only four with duration greater than 30 years had at-risk microalbuminuria.

4.4 Discussion

It appears from these results that, in this clinic population, abnormal albumin excretion is common, 28.6% of all patients having an AER outwith the normal range. Although AER was higher in normal adult males compared to females (chapter 3), no allowance for sex has been made in any of the papers examining the levels of AER predictive of end-stage diabetic nephropathy(19,37,45,46,47,48). For this reason one combined normal range has been used for the current population, regardless of sex.

Comparison of the prevalence of abnormal AER shown here with other papers is difficult, because of differences in populations studied, in the type of urine collections and methods used for measuring albumin. In one large study of an unselected diabetic out-patient clinic population in Denmark, using albumin concentrations in an early morning urine sample, 23% had albumin concentrations $>30 \text{ mg}/\text{l}$, with 11.9% exhibiting concentrations $>30 \text{ mg}/\text{l}$ but being Albustix negative (95). No distinction was made between IDDM and NIDDM, although albumin concentrations $>30 \text{ mg}/\text{l}$ were less common in those patients whose diabetes was diagnosed after the age of 50 years. In another recently published Danish study of 957 IDDM patients, 22% had AER 20-200 $\mu\text{g}/\text{min}$ in a 24h collection and 19% AER $>200 \mu\text{g}/\text{min}$ (96). A British community

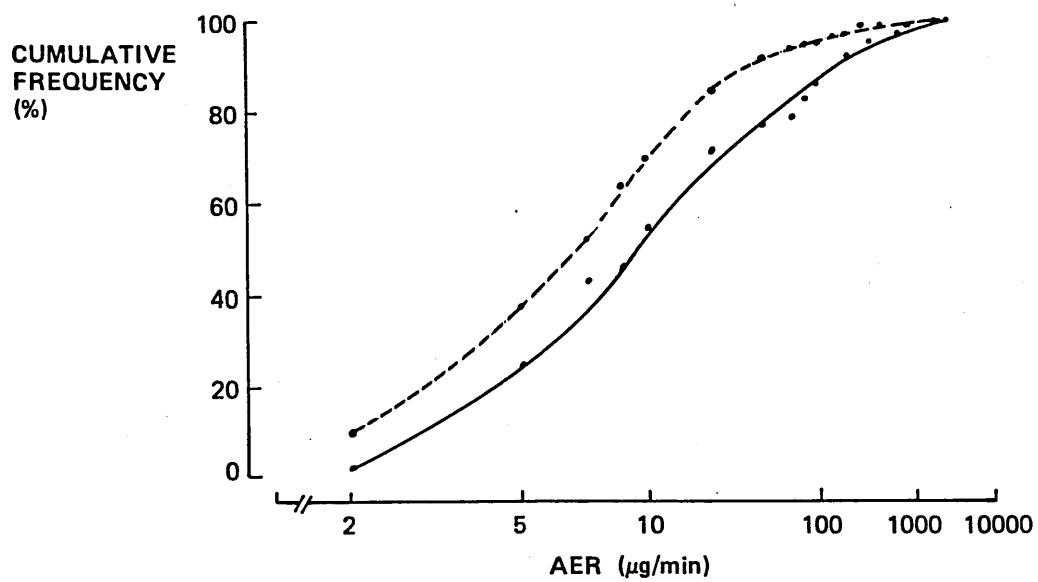


Figure 21 Influence of retinopathy on albumin excretion in NIDDM.

retinopathy —————
 no retinopathy - - - - -

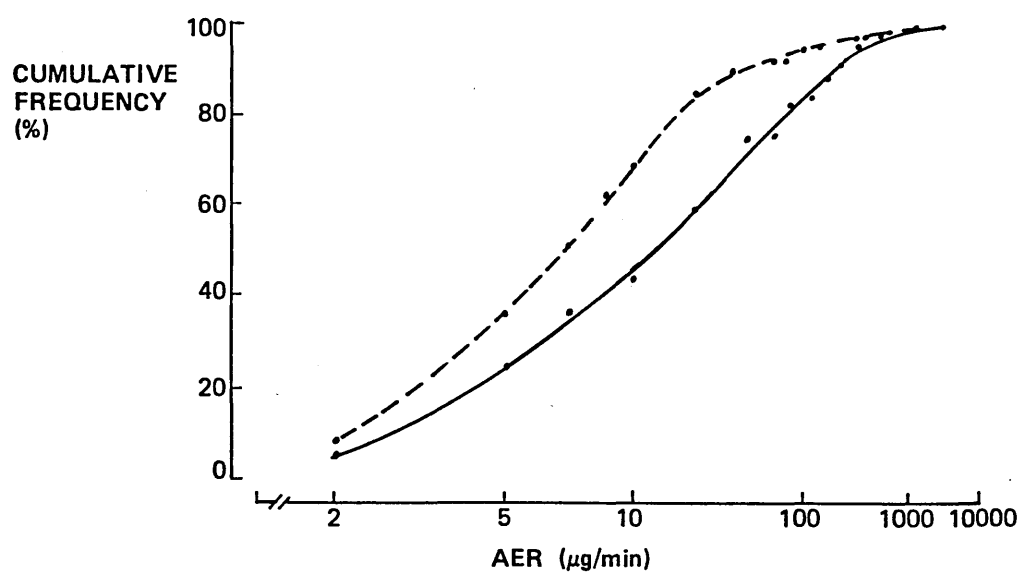


Figure 22 Influence of peripheral vascular disease on albumin excretion in NIDDM.

peripheral vascular disease ———

no peripheral vascular disease - - -

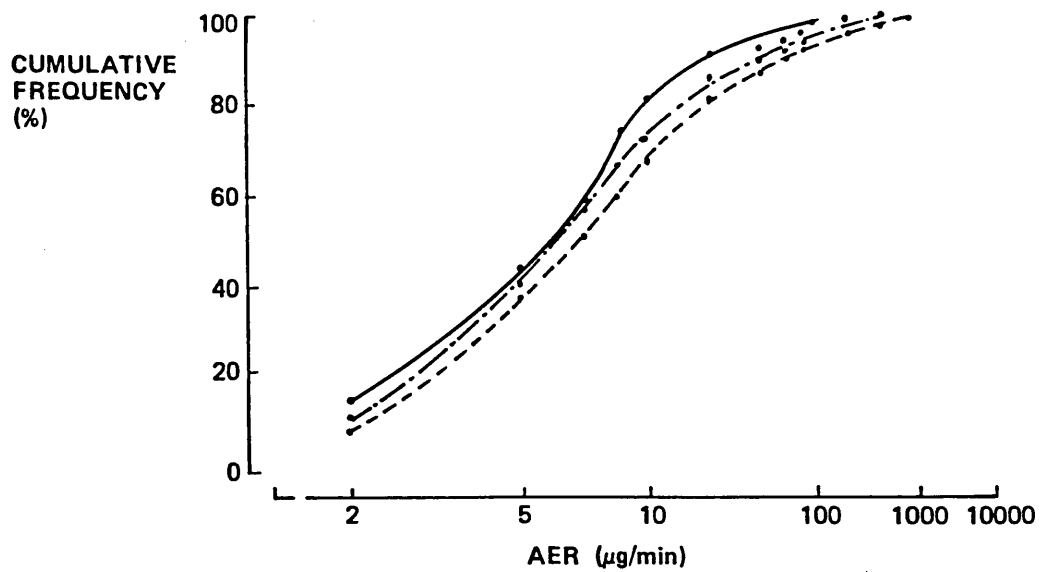


Figure 23 Influence of systolic blood pressure on albumin excretion in NIDDM.

120 mm Hg —————
 140 mm Hg - - - - -
 220 mm Hg — — — — —

based study has recently reported a prevalence of at-risk microalbuminuria (AER >30 µg/min in a timed overnight sample) of 7% in 450 patients and a prevalence of 5.1% of Albustix positive proteinuria(97).

Two British studies have demonstrated a prevalence of persistent Albustix positive proteinuria of 9.4% in insulin-treated patients(51), and 2.7% in all patients(50), whilst an American group found a prevalence of 8.2% at diagnosis of diabetes, rising to 24.6% after 20 years(98). These results suggest that both microalbuminuria and persistent proteinuria may be commoner in Denmark and the USA than in Britain. Whether the differences are real, or simply a reflection of population bias or differences in albumin analysis, remains to be seen. Real differences might perhaps indicate different genetic susceptibilities to diabetic nephropathy or environmental influences such as diet.

In our clinic population, abnormal albumin excretion is commoner in NIDDM than in IDDM, one-third of patients with non-insulin dependent diabetes having abnormal albumin excretion compared with 22 per cent in IDDM. Both mean systolic and diastolic blood pressure were higher in the NIDDM group than the IDDM group and more NIDDM patients were taking anti-hypertensive agents. Systolic blood pressure was significantly related to AER in NIDDM. Thus it may be that the increased prevalence of abnormal AER in NIDDM is related to higher blood pressure, producing increased albumin excretion but without specific diabetic renal disease. Non-diabetic renal disease is thought to be commoner in NIDDM than in IDDM(99) and hypertension per se is known to increase albumin excretion(100).

Although NIDDM patients were older than IDDM patients, no effect of age on AER was observed in either diabetic group or in the normal subjects. It therefore seems unlikely that the higher AER in the NIDDM patients was due to increasing age. Blood glucose control, as measured by glycosylated haemoglobin, was similar in the two groups of patients and had no influence on AER and so is also unlikely to account for the difference between AER in IDDM and NIDDM. A recently published study, by contrast, found no influence of type of diabetes on AER but found positive associations with blood glucose, sex, systolic blood pressure and smoking but not with retinopathy(101). These differences are perhaps explained by the fact that in this

study, patients with Albustix positive proteinuria were excluded from analysis and AER was corrected for body surface area.

At-risk microalbuminuria (AER 30–150 $\mu\text{g}/\text{min}$) occurred in NIDDM at all durations of diabetes, whereas in IDDM only one patient with duration <5 years had such an excretion rate. It is well recognised that, in NIDDM, specific diabetic complications, particularly retinopathy may be present at diagnosis of diabetes, presumably because of the long silent phase of NIDDM. Our data suggest that abnormal albumin excretion, a marker of early renal damage, may also be detectable shortly after diagnosis of NIDDM. Since no patient was studied within 6 months of diagnosis, this is unlikely to be the transiently increased albumin excretion seen with severely uncontrolled diabetes(42,43,102,103,104), but perhaps reflects the fact that many of these patients may have had diabetes for a considerable time before diagnosis, and that significant, permanent renal damage occurred during this period.

The presence of retinopathy strongly influenced AER in both IDDM and NIDDM, whereas male sex was significantly related only in IDDM and systolic blood pressure and peripheral vascular disease in NIDDM. The association of retinopathy and Albustix-positive proteinuria has long been recognised(7,20) and it has been demonstrated that those IDDM patients with AER >15 $\mu\text{g}/\text{min}$ are more likely to progress to proliferative retinopathy as well as to persistent proteinuria(37). Our findings suggest that NIDDM patients with abnormal albumin excretion are also at risk of retinopathy. The close link of retinopathy to albumin excretion in both IDDM and NIDDM suggests a common aetiological mechanism dependent on the diabetic state. In NIDDM, AER in the range 30–150 $\mu\text{g}/\text{min}$ is predictive of early death(48) from cardiovascular disease(19). Thus it is not surprising that AER is closely related to systolic blood pressure and the presence of peripheral vascular disease in NIDDM. Abnormal albumin excretion in NIDDM may perhaps be due to reno-vascular disease rather than to specific diabetic renal disease.

Although the associations with AER shown here are statistically significant, the correlation coefficients are small and account for only a small proportion of the variability in AER. One possible explanation is the large day-to-day variation in AER, some individuals having a coefficient of variation in AER of 40 per cent(105). This was a cross-sectional study and thus may not truly represent preceding events in

the development of diabetic complications. Other factors, not identified here, such as genetics, blood lipids or cigarette smoking, may also influence AER.

In summary, an increased incidence of abnormal albumin excretion in NIDDM compared with IDDM has been shown. At-risk microalbuminuria occurred shortly after diagnosis in NIDDM but was rare in IDDM until after 5 years of diabetes. The features significantly influencing AER were different in NIDDM and IDDM, retinopathy being strongly associated in both groups but male sex only in IDDM and systolic blood pressure and peripheral vascular disease in NIDDM. This is perhaps a reflection of the different risks imposed by microalbuminuria on the 2 groups, IDDM patients being at increased risk of end-stage renal disease whilst NIDDM patients are at risk of early mortality from cardiovascular causes.

CHAPTER 5

Albumin excretion in children with insulin-dependent diabetes

5.1 Aims

The overnight albumin excretion rates in a group of children with IDDM have been compared with AER in normal children and healthy adults. In both groups of children albumin:creatinine ratios have also been measured. The relationship of AER to age and sex in the diabetic children has been explored.

5.2 Subjects and methods

Eighty two diabetic children were requested by post to collect a timed overnight urine sample in connection with their annual "birthday check". A container and written instructions were posted to the parents, asking for the sample to be collected on the night prior to the clinic visit. The age, sex, duration of diabetes and any drug treatment were noted. The presence or absence of retinopathy and the blood pressure (sitting, diastolic phase V) were recorded by one of two observers. Blood was drawn on the same day for determination of glycosylated haemoglobin.

Urinary albumin and creatinine were measured and results expressed as AER ($\mu\text{g}/\text{min}$) and albumin:creatinine ratio (mg/mmol). The results in the diabetic children were compared with those in healthy children and adults described in Chapter 3.

Statistical methods

Albumin excretion rates and albumin:creatinine ratios were positively skewed even after logarithmic transformation in the two groups of children. Results have therefore been analysed by non-parametric tests, using the Kendall Rank and Partial Rank correlation coefficients and Mann Whitney U test.

5.3 Results

Of the 82 children approached, 68 provided a timed overnight urine sample, giving a response rate of 83%: 41 boys and 27 girls were studied. Apart from 1 boy who had had bilateral cataract extractions, none had evidence of the long-term complications of diabetes. All were Albustix negative on routine clinic testing of the urine. The mean duration of diabetes was 3.8 years (range 1-13) and HbA_{1c} 9.9% (5.6-14.3). Mean AER was similar to that of the non-diabetic children and the groups were well matched for age and sex (Table 14). AER correlated with age ($r=0.36$, $p<0.001$; Figure 24), as it

	Normal Children			Diabetic Children		
	All	Male	Female	All	Male	Female
Number	64	32	32	68	41	27
Age	9.0	8.0	9.9	10.3	9.3	11.9**
(years)	(3-17)	(3-17)	(4-15)	(3-17)	(4-15)	(3-17)
HbA ₁	-	-	-	9.9	9.7	10.1
(%)				(5.6-14.3)	(5.6-13.7)	(5.7-14.3)
Duration of	-	-	-	3.8	3.7	4.1
diabetes						
(years)				(1-13)	(1-13)	(1-10)

Mean (range)

**p<0.005 diabetic girls v boys

Table 14 Characteristics of the normal and diabetic children studied

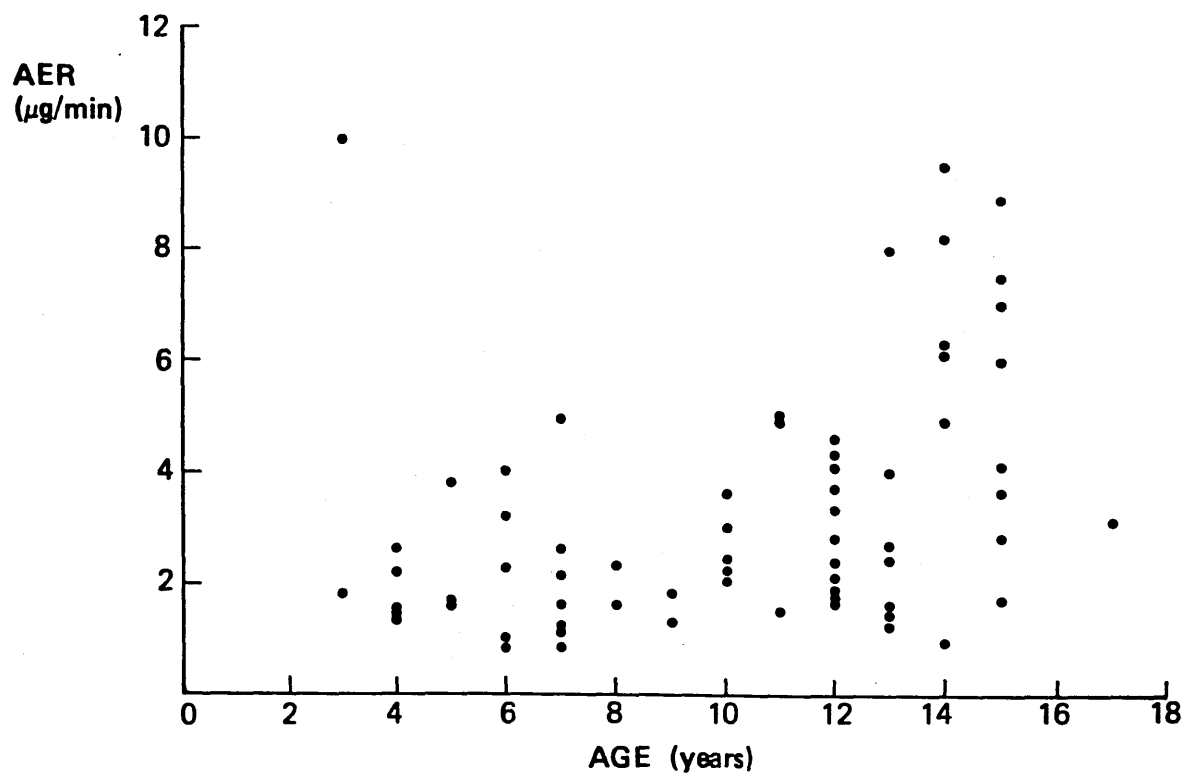


Figure 24 Albumin excretion in diabetic children: effect of age

$r = 0.36, p < 0.001$

did in the non-diabetic children, and also weakly with duration of diabetes ($\gamma=0.22$, $p<0.01$), but when controlled for age this became insignificant. There was no correlation of AER with HbA_{1c}. AER was significantly lower in both groups of children than in the adult population ($p<0.001$; Table 15).

Albumin excretion rates in diabetic girls were higher than in boys [3.6(0.9–9.5) versus 2.1(0.7–8.2) $\mu\text{g}/\text{min}$; $p=0.02$; Table 15]. However, the girls were significantly older than the boys [11.9(3–17) versus 9.3(4–15) years; $p=0.005$]. It was possible to match 23 female diabetic children (85%) with boys to within 2 years for age and duration of diabetes, so that there was no difference between the mean age [10.9(4–17) versus 11.1(4–15) years] or duration [3.7(1–10) versus 3.7(1–12) years] of the groups. There was no significant difference in AER between these groups [3.1(0.9–9.5) versus 2.4(1.1–7.5) $\mu\text{g}/\text{min}$; $t=1.49$, $p>0.1$] suggesting that the higher excretion rate observed in the total population of diabetic girls was due to the greater age.

Albumin:creatinine ratios were similar to the non-diabetic children [1.00(0.43–2.58) versus 0.95(0.40–3.7) mg/mmol] and did not correlate with age, HbA_{1c} or duration of diabetes.

5.4 Discussion

The results demonstrate that albumin excretion rates are significantly lower in children than adults and that the excretion rate increases with age in both normal and diabetic children. Two previous studies(53,56) have failed to demonstrate any effect of age on normal children but in a large study of 374 normal children(58) both day and night albumin excretion rates correlated strongly with age. Similarly, in diabetic children, the published data is unclear. Davies(54) and Rowe(53) demonstrated a relationship with age but Ellis(56) and Brochner-Mortensen(57) failed to find it. None of our diabetic children had albumin excretion rates in the adult microalbuminuric range.

Direct comparison of albumin excretion rates between studies is difficult because of differences in sample collection and in albumin assay techniques. We have chosen to collect timed overnight urine samples in an attempt to minimise the effect of the exaggerated albumin excretion response to exercise observed in childhood(59,106,107), and adult diabetes(60,82). Two studies have found an increased overnight excretion rate in diabetic over normal children(53,54) which we did not. Our children however

AER (µg/min)			
	n	median	range
Adults	106	4.3	1.6-17.6
Non-diabetic children			
Total	64	2.4	0.2-20.8
Boys	32	2.2	0.2-20.8
Girls	32	2.4	0.2-10.6
Diabetic children			
Total	68	2.5	0.7-9.5
Boys	41	2.1	0.7-8.2
Girls	27	3.6*	0.9-9.5

* p=0.02 diabetic girls v boys

Table 15 Albumin excretion rates in diabetic and non-diabetic children and normal adults

were younger and had diabetes of shorter duration. These studies also demonstrated a significant relationship between AER and duration of diabetes.

The literature examining the correlation of albumin excretion rate with metabolic control is confusing. We have shown no correlation of HbA_{1c} with albumin excretion rate, a finding observed previously(55,56). However, Davies demonstrated significant relationships of AER with HbA_{1c}, mean blood glucose and M values(54) and Rowe with HbA_{1c}(53). It has also been demonstrated that the exaggerated albumin excretion in response to exercise in diabetic children can be decreased by improving metabolic control(106). Microalbuminuria in adults can be reduced by the institution of normoglycaemia in 24 hours(102). Since the half-life of HbA_{1c} is approximately 6 weeks, a close correlation between AER and HbA_{1c} might not be expected.

In conclusion, AER is significantly lower in normal and diabetic children than in adults and correlates with age in all children. It is therefore unlikely that the cut-off points suitable for defining microalbuminuria in adults can be applied to children. A long-term follow-up study is needed to determine the natural history of the development of microalbuminuria in children and the cut-off limits predictive of future diabetic nephropathy.

Since this study was completed, a further paper has shown a prevalence of 37% of microalbuminuria in insulin-dependent diabetic children aged over 15 years(108). No children under the age of 15 had microalbuminuria.

The absence of children with abnormal albumin excretion, so that all the excretion rates were within the rather narrow normal range may also have contributed to the lack of correlation between AER and HbA_{1c} demonstrated here.

CHAPTER 6

Day-to-day and longer term variation in albumin excretion

6.1 Aims

This study was undertaken to evaluate the day-to-day variation of albumin excretion in normal subjects and diabetic patients with varying degrees of albuminuria and to observe the rate of change of albumin excretion in normoalbuminuric and microalbuminuric diabetic subjects over a longer time.

6.2 Subjects and Methods

(a) Day-to-day variation

Eight normal subjects and 21 patients with IDDM collected a minimum of 5 timed overnight urine samples in 6 weeks. The diabetic patients were characterised on the basis of their mean AER:

Group 1 : AER $\leq 10.5 \mu\text{g}/\text{min}$

Group 2 : AER $10.6\text{--}150 \mu\text{g}/\text{min}$

Group 3 : AER $>150 \mu\text{g}/\text{min}$

Characteristics of the subjects are given in Table 16. No subject took any medication other than insulin during the study and all remained healthy over the 6 weeks. Coefficients of variation of AER were calculated for each patient.

(b) Long-term follow-up

Ten insulin-dependent diabetic patients with normal AER collected one timed overnight urine sample every 6 weeks for 60 weeks. Eight IDDM patients with normoalbuminuria and 9 with AER $10.6\text{--}150 \mu\text{g}/\text{min}$ collected a minimum of one urine sample every 6 months for at least 18 months.

6.3 Results

(a) Day-to-day variation

Table 16 gives the characteristics of the subjects studied and the mean coefficients of variation for the normal and the 3 diabetic groups. The mean CV in each group is high, and there is a wide fluctuation in the variation, as seen by the large range of the CV's. However, all normal subjects remained within the normal range. In the normoalbuminuric diabetic group (group 1), 2 subjects on 1 occasion and 1 subject on

	Normal subjects	Diabetic subjects		
		Group 1	Group 2	Group 3
Number	8	9	6	6
Age (years)	30.9 (26-34)	34.7 (23-46)	47.2 (37-54)	33.0 (21-46)
Sex (M:F)	5:3	5:4	5:1	5:1
Number of urine collections (range)	9.4 (7-11)	5.9 (5-6)	6 (6)	6 (6)
CV of AER (%)	13.1 (8.3-16.9)	47.2 (10.8-73.3)	17.0 (4.7-52.9)	10.5 (5.7-16.1)
Initial AER ($\mu\text{g}/\text{min}$)	4.1 (3.0-5.6)	3.3 (2.3-6.9)	34.2 (16.6-116.1)	366 (221-1558)

Mean (range)

AER: albumin excretion rate

CV: coefficient of variation

Table 16 Day-to-day variation in albumin excretion in normal and diabetic subjects.

2 occasions had AER $>10.5 \mu\text{g}/\text{min}$. In group 2, 1 subject on 1 occasion had AER $<10.5 \mu\text{g}/\text{min}$. In the macroalbuminuric group (group 3) 1 subject on 1 occasion and 2 subjects on 2 occasions had AER $<150 \mu\text{g}/\text{min}$.

(b) Longer term follow-up

Figure 25 illustrates the change in AER measured every 6 weeks for 60 weeks in a group of 10 IDDM patients initially with normal AER. The mean change in AER over the 60 weeks was $+44.6 (-58.6 - +125.0)\%$ of the initial value [mean(range)]. One subject showed fluctuations in AER outwith the normal range, AER increasing from 8.1 to $17.4 \mu\text{g}/\text{min}$ during the 60 weeks. In the other 9 patients, AER was always $<10.5 \mu\text{g}/\text{min}$.

Follow-up for a longer period of time (18 to 30 months) revealed rates of changes in AER [mean % change(range)] of $+11.3(-31.4 - +105.5)\%$ per year in normoalbuminuric and $+1.3 (-44.4 - +101.8)\%$ per year in microalbuminuric patients. Figure 26 illustrates the results for all patients and Figure 27 representative individual changes. One normoalbuminuric patient became microalbuminuric (AER from 7.9 - $18.9 \mu\text{g}/\text{min}$ in 30 months), one microalbuminuric became normoalbuminuric (initial AER $40.1 \mu\text{g}/\text{min}$, 3 out of 4 subsequent measurements $<10.5 \mu\text{g}/\text{min}$) and 2 became macroproteinuric (initial AER's 132 and $80 \mu\text{g}/\text{min}$).

6.4 Discussion

The high day-to-day variability in AER in both normal subjects and diabetic patients with all degrees of albuminuria has major implications for studies investigating the effect of various therapeutic manoeuvres on albumin excretion. Multiple urine collections are necessary to ensure that any change in AER observed is due to a "true" change and not simply natural variation. There are many known causes for varying albumin excretion, including posture(42,54,58), exercise(59,60), hydration and dietary protein intake(61). In addition, in the diabetic person, acute changes in blood glucose concentrations may be reflected by changes in AER(42,43,102).

The risk to those diabetic patients fluctuating from normal to abnormal AER is unknown. It has long been appreciated that persistent Albustix positive proteinuria is preceded by a stage of intermittent proteinuria. It is possible that a similar stage of "intermittent microalbuminuria" exists before the higher AER levels become persistent.

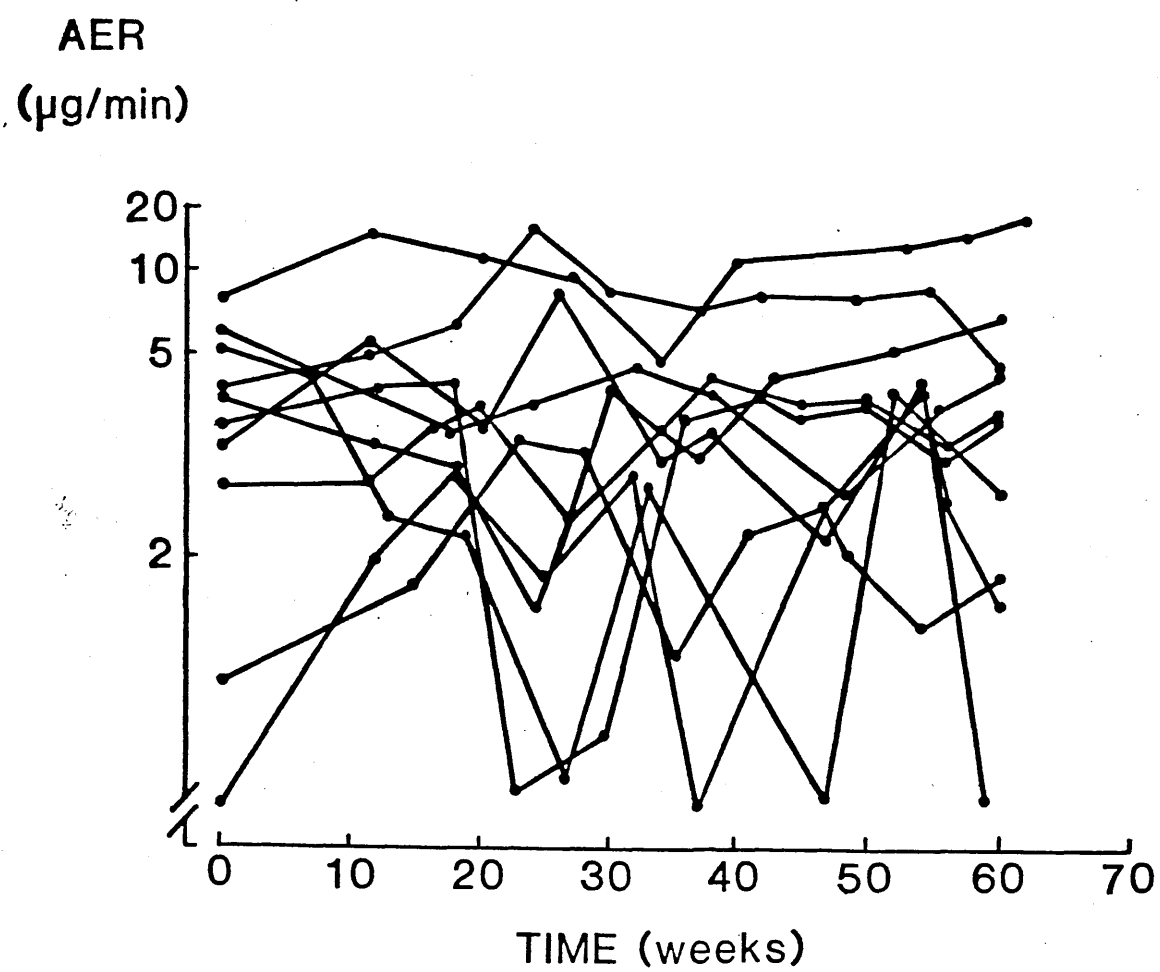


Figure 25 Long-term changes in albumin excretion rates (AER) in 10 IDDM patients with initially normal albumin excretion

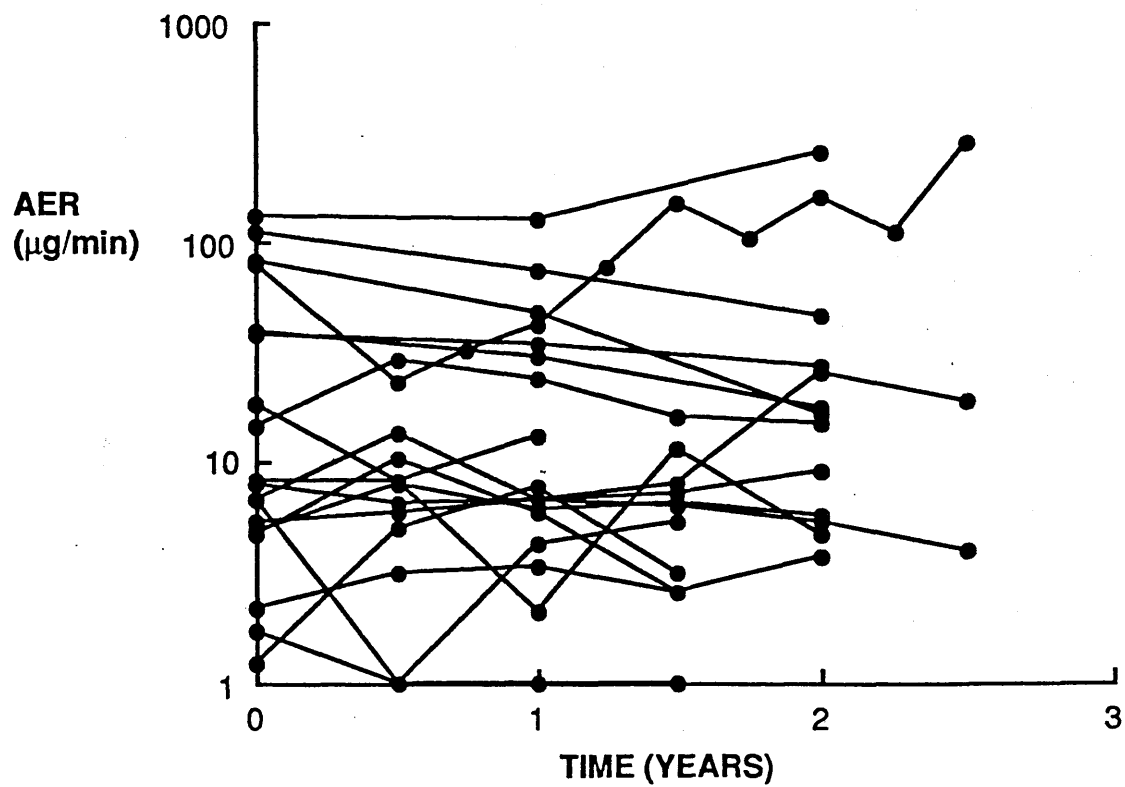


Figure 26 Long-term changes in albumin excretion rates (AER) in 8 normoalbuminuric and 9 microalbuminuric IDDM patients.

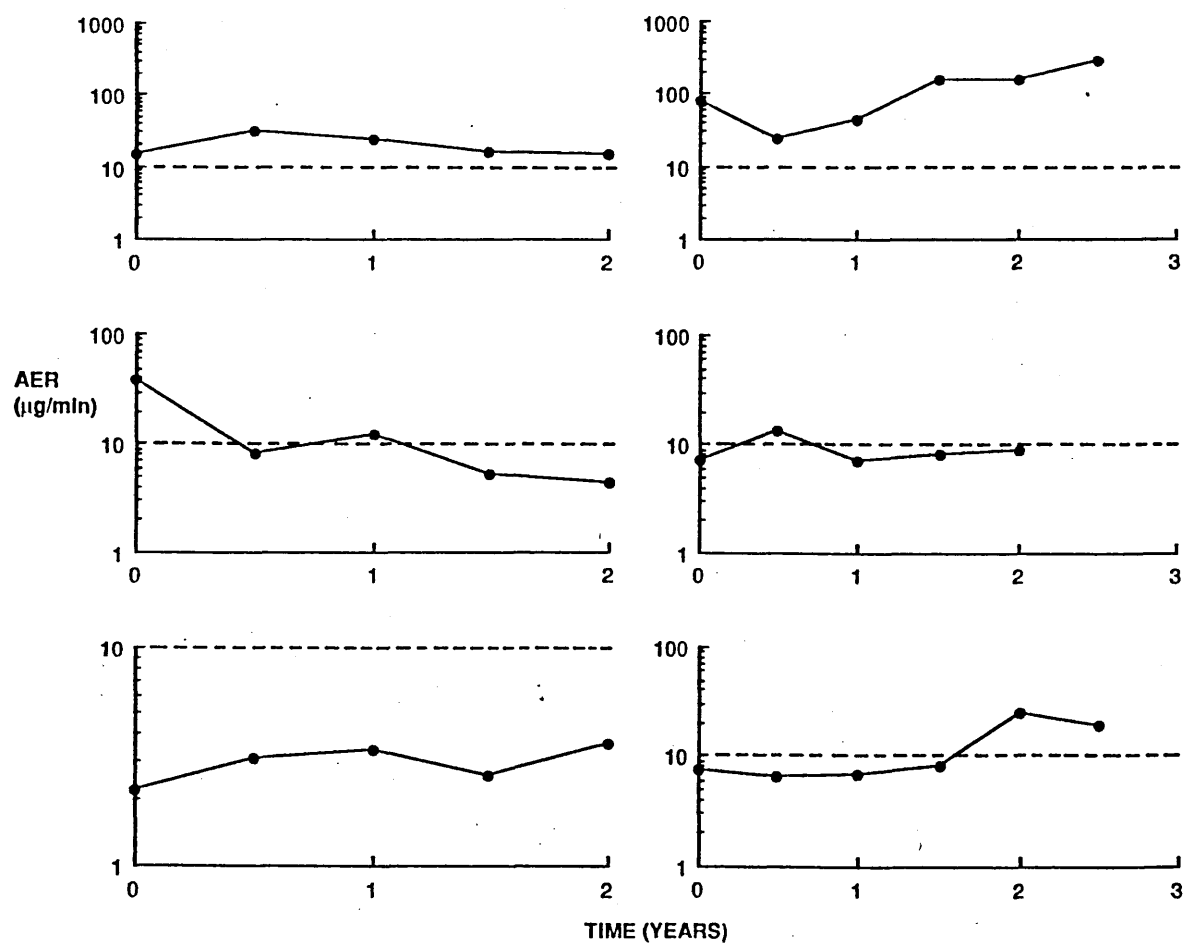


Figure 27 Changes in albumin excretion rate (AER) in 6 IDDM patients.

----- Upper limit of normal AER.

Obviously, multiple testing is required before an individual is assigned to a particular stage of diabetic nephropathy. However, it is noteworthy that several of the studies showing that microalbuminuria is predictive of clinical nephropathy were based on single urine collections(45,46,47,48).

Little information is available on the rate of change of albumin excretion in the progression either from normoalbuminuria to microalbuminuria or microalbuminuria to clinical proteinuria. The data presented here suggests that the mean rate of change is rather slow, although there is a wide variation between individuals. In the studies showing that microalbuminuria predicts the later development of clinical proteinuria, the shortest follow-up time was 6 years(46,47) and the longest 14(45). In a longitudinal study of 10 IDDM patients with elevated albumin excretion followed for a mean of 4.9 years, the mean increase in AER was 19.8% per year, but with a range of -7.3 to 58.5% per year(109). Such a wide variation in rate of progression mimics that seen in persistent proteinuria, where although the rate of decline in renal function is constant for any individual patient, large variations exist between patients(9,20,21,110). In any trial of therapeutic intervention, the effect of this large variability must be allowed for.

A recently published small study observing albumin excretion in both IDDM and NIDDM patients over a mean of 7 years, confirmed that transient episodes of micro- and macroalbuminuria often occur before micro- or macroalbuminuria become persistent(111). In this study, the transition time from microalbuminuria to persistent proteinuria varied from 3 to 5 years, a considerably shorter time than has previously been thought.

It is thus imperative that large natural history studies are completed and that intervention studies have sufficient power to allow for wide intra- and inter individual variation in changes in albumin excretion.

In the study described here, and in the previously reported papers, the numbers of patients studied and the observations made were rather small. This may account, at least in part, for the large variations in rate of change of AER.

CHAPTER 7

The effect of Bromhexine on albumin excretion in insulin-dependent diabetes

7.1 Aims

The aim of this study was to examine the effect of the mucolytic agent bromhexine on albumin excretion in the various stages of albuminuria in IDDM.

7.2 Subjects and methods

Subjects

Twenty one IDDM patients were studied. They were divided into 3 groups on the basis of overnight albumin excretion rates in at least 2 out of 3 sterile, ketone-free urine collections:

Group 1 : normoalbuminuric, AER $\leq 10.5 \mu\text{g}/\text{min}$; n=9

Group 2 : microalbuminuric, AER 10.6–150 $\mu\text{g}/\text{min}$; n=6

Group 3 : macroalbuminuric, AER $>150 \mu\text{g}/\text{min}$; n=6

Patient characteristics are shown in Tables 17 and 18. Those in group 2 were older than in group 1 [47(37–54) versus 35(23–46) years; mean (range); $p<0.01$] and group 3 [33(21–46) years; $p<0.01$]. Systolic blood pressure was higher in groups 2 and 3 compared to group 1 [137(118–142) and 141(132–146) versus 122(112–142) mmHg; $p<0.01$ and $p<0.001$]. Diastolic blood pressure was higher in group 3 compared with group 1 [87(80–94) versus 79(72–90) mmHg; $p<0.05$]. One patient in group 3 took carbamazepine for painful peripheral neuropathy and thyroxine for auto-immune thyroid disease, in constant dosage throughout the study. No other subject took any medication other than insulin during the study and diet was unaltered.

Study design

cross-over

The study was a placebo-controlled, double-blind/cross-over randomised trial. Subjects were randomised at baseline, within their albumin excretion groups, to receive bromhexine 8 mg or placebo tablets, three tablets three times a day, each for 4 weeks. There was a 2 week "wash-out" period between each treatment period when all patients took placebo tablets. Clinical and laboratory measurements were made at the beginning and end of each treatment period. Patients collected 3 timed overnight urine samples for measurement of albumin and β_2 -microglobulin at each time point. Blood was drawn for determination of HbA_{1c}, serum β_2 -microglobulin, fructosamine, and plasma creatinine. Blood pressure was measured in the sitting position after 5 minutes rest,

	1	Group 2	3
Age (years)	35 (23-46)	47** (37-54)	33 ⁺⁺ (21-46)
Duration diabetes (years)	16 (6-29)	23 (7-32)	24 (19-31)
Retinopathy (Background : Proliferative)	3:0	4:2	3:3
HbA ₁ (%)	8.1 (6.6-10.7)	10.6* (8.1-13.1)	10.2* (8.6-11.7)
Fructosamine (mmol/100g albumin)	3.05 (2.55-3.78)	3.09 (2.63-3.55)	3.23 (2.79-3.56)
Systolic Blood Pressure (mmHg)	122 (112-142)	137** (118-142)	141*** (132-146)
Diastolic Blood Pressure (mmHg)	79 (72-90)	81 (80-84)	87* (80-94)

Mean (range)

*, **, *** p<0.05, p<0.01, p<0.001 versus group 1

⁺⁺ p<0.01 group 3 versus group 2

Table 17 Effect of bromhexine on albumin excretion in IDDM:
Patient characteristics at baseline.

	GROUP		
	1	2	3
AER ($\mu\text{g}/\text{min}$)	3.2 (2.1-8.8)	36*** (22-96)	321 ⁺⁺⁺ *** (202-1216)
β_2 -M excretion rate (ng/min)	28 (7-85)	68 (4-541)	33 (14-210)
Serum β_2 -M (mg/l)	1.4 (1.2-1.9)	1.8 (1.5-2.0)	1.8 (1.5-2.0)
Serum creatinine ($\mu\text{mol}/\text{l}$)	89 (59-118)	87 (68-118)	97 (82-124)

β_2 -M : β_2 -Microglobulin

*** $p < 0.001$ versus group 1

+++ $p < 0.001$ versus group 2

Table 18 Effect of bromhexine on albumin excretion in IDDM:
Renal characteristics at baseline

using Korotkoff phase V diastolic pressure. A tablet count was performed to check compliance. On the day prior to each clinic visit, patients performed 4 capillary blood glucose tests using BM Test Glycemie 1-44 strips (Boehringer, Mannheim, West Germany), one test before each main meal and one before bed. The test strips were returned to an air-tight container and brought to hospital next day, where they were read by reflectance meter.

Statistical analysis

Results were analysed at the end of each treatment period, irrespective of order, and also at each time point, irrespective of treatment, to look for an order effect. The mean of the 3 albumin and β_2 -microglobulin excretion rates at each time point was calculated to allow for day-to-day variation. Albumin and β_2 -microglobulin excretion rates were log transformed before analysis to correct for skewed distribution. The differences within and between the groups was assessed by paired or unpaired Student's t test, with correction for multiple testing (112). Results are expressed as mean (range).

7.3 Results

Analysis for the results of each time point revealed no order effect in any variable. Albumin excretion rates were similar after 1 month's treatment with bromhexine or placebo in all 3 groups (Table 19 and Figure 28). Similarly, other measures of renal function were not different between treatment periods (Table 19). The other variables of blood pressure and blood glucose known to effect albumin excretion remained constant in all 3 groups throughout the study (Table 20 and Figure 29). A tablet count revealed that patients had taken 97.3 (84.9-100)% of bromhexine and 95.5(93.7-100)% of placebo tablets. No adverse effects of treatment were noted.

7.4 Discussion

No effect of treatment with bromhexine 72 mg daily for one month on albumin excretion or any other measure of glomerular or renal tubular function in IDDM patients with varying degrees of albuminuria has been demonstrated. Nor was there any change in blood glucose control or blood pressure, variables which by themselves alter albumin excretion. The hallmark of diabetic nephropathy is accumulation of abnormal glycoproteins in the basement membrane and mesangium(62,65), reducing the glomerular filtration surface, with a strong correlation of glomerular filtration rate and

	GROUP 1		GROUP 2		GROUP 3	
	Placebo	Bromhexine	Placebo	Bromhexine	Placebo	Bromhexine
AER ($\mu\text{g}/\text{min}$)	3.3 (1.9-13.2)	3.6 (1.7-13.5)	37 (20-103)	40 (21-129)	443 (292-2593)	397 (248-2160)
β_2 -M Excretion rate (ng/min)	28 (8-51)	19 (1-113)	39 (11-101)	44 (2-201)	59 (25-423)	53 (31-172)
Serum β_2 M (mg/l)	1.4 (0.9-1.9)	1.7 (1.1-2.0)	1.8 (1.5-2.0)	1.9 (1.6-2.1)	2.0 (1.6-3.0)	1.8 (1.5-2.1)
Serum creatinine ($\mu\text{mol}/\text{l}$)	86 (77-108)	85 (80-103)	91 (78-115)	95 (85-115)	110 (86-180)	96 (81-118)

β_2 -M : β_2 -Microglobulin

Mean (range)

Table 19 Effect of bromhexine on albumin excretion in IDDM: renal function after one months treatment with bromhexine and placebo.

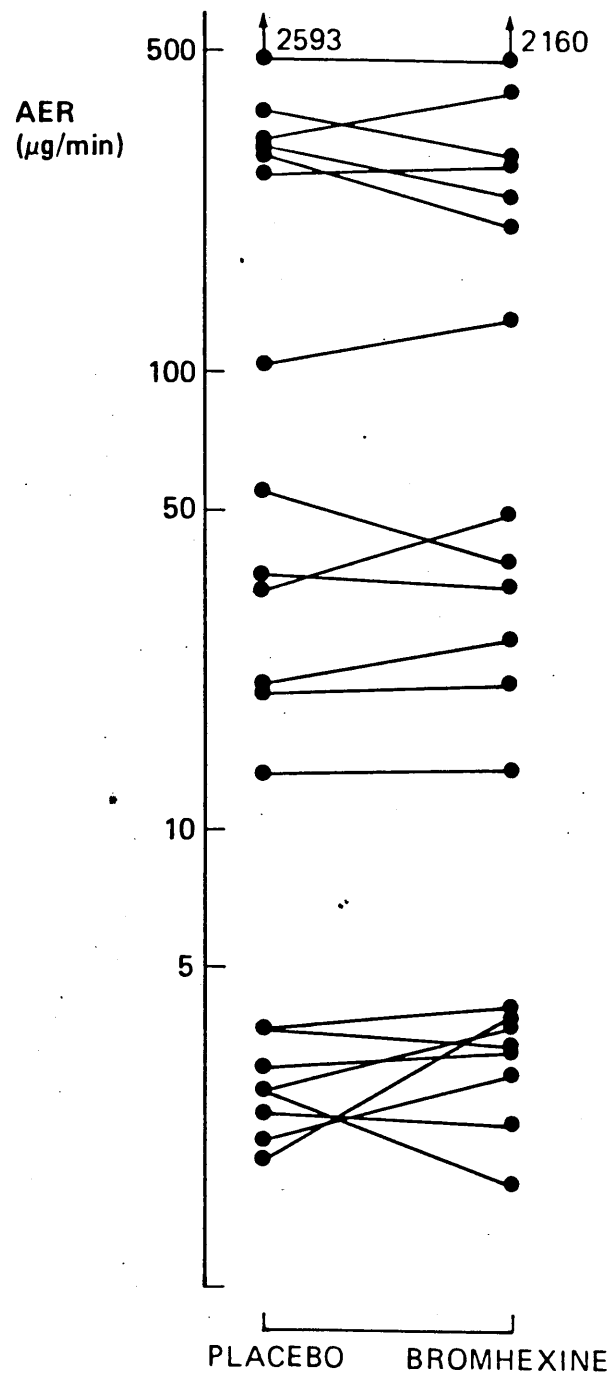


Figure 28 Albumin excretion rates in 21 IDDM patients after one month's treatment with bromhexine or placebo.

	GROUP 1		GROUP 2		GROUP 3	
	Placebo	Bronhexine	Placebo	Bronhexine	Placebo	Bronhexine
Fructosamine (mmol/100g albumin)	3.08 (2.55-3.34)	2.95 (2.42-3.30)	3.07 (2.81-3.38)	3.09 (2.63-3.58)	3.36 (3.19-3.60)	3.34 (3.14-3.58)
HbA _{1c} (%)	7.8 (6.1-10.3)	8.3 (6.3-10.2)	10.1 (8.0-11.4)	9.2 (6.3-12.9)	10.3 (9.1-11.7)	9.2 (7.2-10.7)
Systolic BP (mm Hg)	120 (110-138)	121 (104-142)	137 (124-150)	135 (126-142)	141 (138-150)	143 (138-150)
Diastolic BP (mm Hg)	78 (70-92)	78 (78-88)	76 (60-86)	80 (70-86)	86 (76-94)	86 (78-90)
Mean (range)						

Table 20 Glycaemic control and blood pressure after one months treatment with bronhexine and placebo.

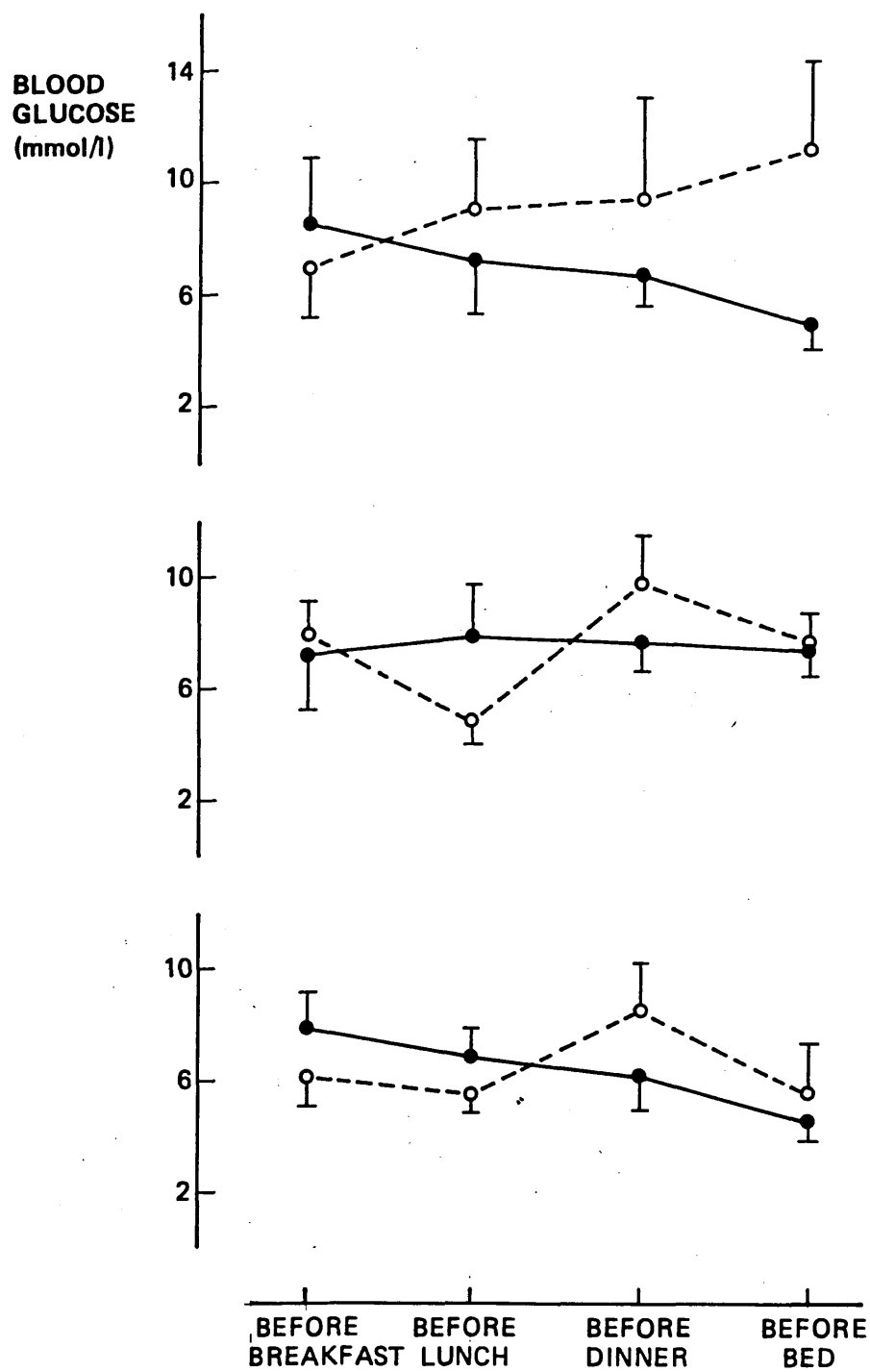


Figure 29 Laboratory read capillary blood glucose concentrations after one months treatment with bromhexine (●—●) and placebo (○—○). Mean (SD).

filtration surface per nephron(63). It might be expected that bromhexine, by its mucolytic action on glycoprotein metabolism, might reduce glycoprotein deposition in the glomerulus, increasing the available filtration surface and improving kidney function. Previous work has shown a decrease in carbohydrate-containing materials in diabetic patients after one months therapy with an identical dose of bromhexine(67). However, albumin excretion was not measured and the patients may all have had normal renal function. It may be that albumin excretion elevated above normal represents irreversible structural damage in IDDM, hence bromhexine would have no influence on albumin excretion. It has been suggested that another major determinant of albumin excretion in IDDM is the loss of anionic charge on the basement membrane, perhaps due to loss of heparan-sulphate proteoglycan, rather than actual accumulation of glycoprotein(66). If bromhexine was ineffective in increasing anionic change on the basement membrane, no effect would be seen on albumin excretion.

In experimental diabetes, bromhexine has been shown to significantly decrease glomerular volume, with trends towards reduction of basement membrane thickening and kidney volume(68). The lack of effect on albumin excretion of one months treatment with bromhexine 72 mg daily in IDDM patients with varying degrees of albuminuria demonstrated here is thus disappointing.

CHAPTER 8

Relationship of urinary albumin excretion to the excretion of human epidermal growth factor

8.1 Aims

Neither the cause of the whole kidney enlargement in early diabetes nor of the later mesangial expansion has been fully elucidated. It is possible that growth factors have some role in these processes. The study examines the pattern of hEGF excretion in healthy subjects and in diabetic patients with varying degrees of albuminuria.

8.2 Methods

Subjects

Nineteen healthy subjects, 11 male and 8 female, (group 1) and fifty five diabetic patients, 29 male and 26 female, were studied. The patients were divided into 3 groups on the basis of albumin excretion rate (AER).

Group 2 : normoalbuminuria; AER $\leq 10.5 \mu\text{g}/\text{min}$

Group 3 : at-risk microalbuminuria; AER 30–150 $\mu\text{g}/\text{min}$

Group 4 : macroalbuminuria; AER $>150 \mu\text{g}/\text{min}$

The characteristics of the patients in each group are shown in Table 21. No subject had a history of malignant or thyroid disease.

Data from patients with IDDM and NIDDM were analysed together because of the small numbers.

Laboratory methods

Each subject collected one timed overnight urine sample for determination of albumin, hEGF and creatinine. AER was calculated from the duration and volume of the urine collection. The urinary hEGF:creatinine ratio was calculated in an attempt to relate hEGF excretion to renal function.

Statistical analysis

AER values were logarithmically transformed before analysis to correct for skewed distribution. The significance of differences between mean values was assessed by Student's t test and correlations by Pearson correlation coefficient.

8.3 Results

There was a wide range in hEGF excretion from 0.20 to 11.8 nmol/l in the total population studied. There was no significant difference in hEGF between the sexes in

	NORMAL GROUP 1	GROUP 2	DIABETIC GROUP 3	GROUP 4
AER (µg/min)	<10.5	<10.5	30-150	>150
Number	19	18	19	18
Sex (M:F)	11:8	4:14	12:7	10:8
Age (years)	30 (23-47)	60*** (29-74)	50*** (23-74)	58*** (28-77)
Duration diabetes (years)		6 (1-29)	13 ¹ (1-29)	12 ² (1-32)
HBA ₁ (%)		9.7 (6.7-15.8)	9.5 (7.0-12.0)	9.5 (6.5-11.7)
Systolic BP (mm Hg)		144 (100-190)	136 (105-200)	159 ² (110-220)
Diastolic BP (mm Hg)		84 (60-100)	80 (65-95)	89 ² (80-100)
IDDM:NIDDM		4:14	10:9	8:10

Mean (range)

*** p<0.001 versus non-diabetic group

¹ p<0.05 group 3 versus 2

² p<0.05 group 4 versus 2

Table 21 Epidermal growth factor excretion and albuminuria: Characteristics of subjects studied.

the whole population or in any of the individual groups studied. Diabetic patients had significantly lower hEGF values than controls, regardless of albumin excretion rate (Table 22). There was no difference in urine creatinine concentrations in males compared to females in any of the groups studied. However, creatinine concentrations were lower in all 3 diabetic groups compared to group 1 (Table 22). There was no difference in creatinine concentrations between the diabetic groups.

Albumin excretion rates were significantly higher in normal males than females [4.8(2.5-6.9) versus 2.6(1.6-9.7) $\mu\text{g}/\text{min}$; $p<0.01$]. However, no influence of sex on AER was seen in any of the diabetic groups. As expected, AER in group 1 was similar to group 2, with significantly higher values in groups 3 and 4 (Table 22).

The hEGF:creatinine ratio was similar in males and females in all the groups. Subjects in groups 1 and 2 had similar ratios, but those in groups 3 and 4 had significantly lower ratios than in groups 1 and 2 ($p<0.001$ for both) and group 4 was significantly different from group 3 ($p<0.001$; Table 22). There was a significant inverse correlation between hEGF:creatinine ratio and AER in the diabetic patients ($r=-0.49$, $p=0.02$, Figure 30). Urinary hEGF excretion correlated powerfully with urinary creatinine in the normal ($r=0.84$, $p<0.001$) and the diabetic population ($r=0.91$, $p<0.001$, Figure 31). There was no relation of hEGF or hEGF:creatinine ratio to age.

8.4 Discussion

Urinary hEGF:creatinine ratios are decreased in diabetic patients with pathological albuminuria, the decrease being related to the degree of albuminuria. The hEGF concentration in the overnight urine collections varied considerably, probably due to differences in urine volume and solute concentrations. It was thus necessary to express hEGF excretion in terms of urinary creatinine excretion. The ratio of hEGF to creatinine has previously been used as an index of urinary hEGF excretion(113). The present results confirm those from a previous study showing a reduced EGF:creatinine ratio in end-stage diabetic nephropathy(114) and extend the observation to patients with lesser degrees of renal impairment.

Reduction of hEGF excretion may be explained either by decreased glomerular filtration or by impaired renal tubular synthesis or excretion. With a molecular weight of approximately 6000 daltons, one tenth that of albumin, hEGF appears freely in the

	NORMAL GROUP 1	GROUP 2	DIABETIC GROUP 3	GROUP 4
AER ($\mu\text{g}/\text{min}$)	3.7 (1.6-9.7)	3.5 (1.4-9.8)	75.2+++ (30.0-127.9)	289.5 ^{†††} +++ (169-879)
Creatinine (mmol/l)	7.5 (2.5-14.7)	3.1+++ (0.8-9.8)	5.0+ (0.8-13.0)	4.7 (0.9-14.3)
hEGF (nmol/l)	4.5 (1.5-11.8)	1.9+++ (0.3-4.9)	2.6+++ (0.3-7.6)	2.1+++ (0.1-7.1)
hEGF/creatinine (nmol/mmol)	0.69 (0.47-1.27)	0.60 (0.16-1.36)	0.47 ^{***} +++ (0.10-0.83)	0.38 ^{***} +++ (0.10-0.63)

Mean (range)

+++,+: $p < 0.001$, $p < 0.05$ versus group 1

*** $p < 0.001$ versus group 2

††† $p < 0.001$ versus group 3

Table 22 Albumin, hEGF and creatinine excretion in normal subjects (group 1) and diabetic patients with normal albumin excretion (group 2), microalbuminuria (group 3) and macroalbuminuria (group 4)

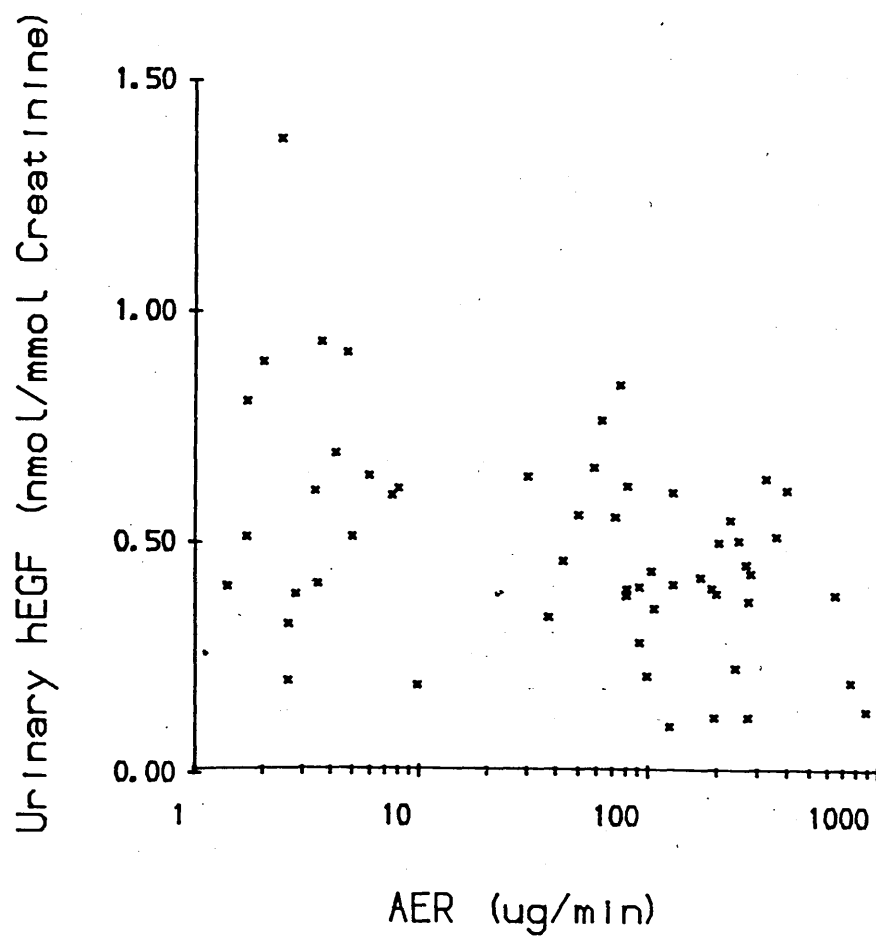


Figure 30 Epidermal growth factor : Relationship of AER to hEGF:creatinine ratio in the diabetic subjects.
 $r=0.49$, $p=0.02$

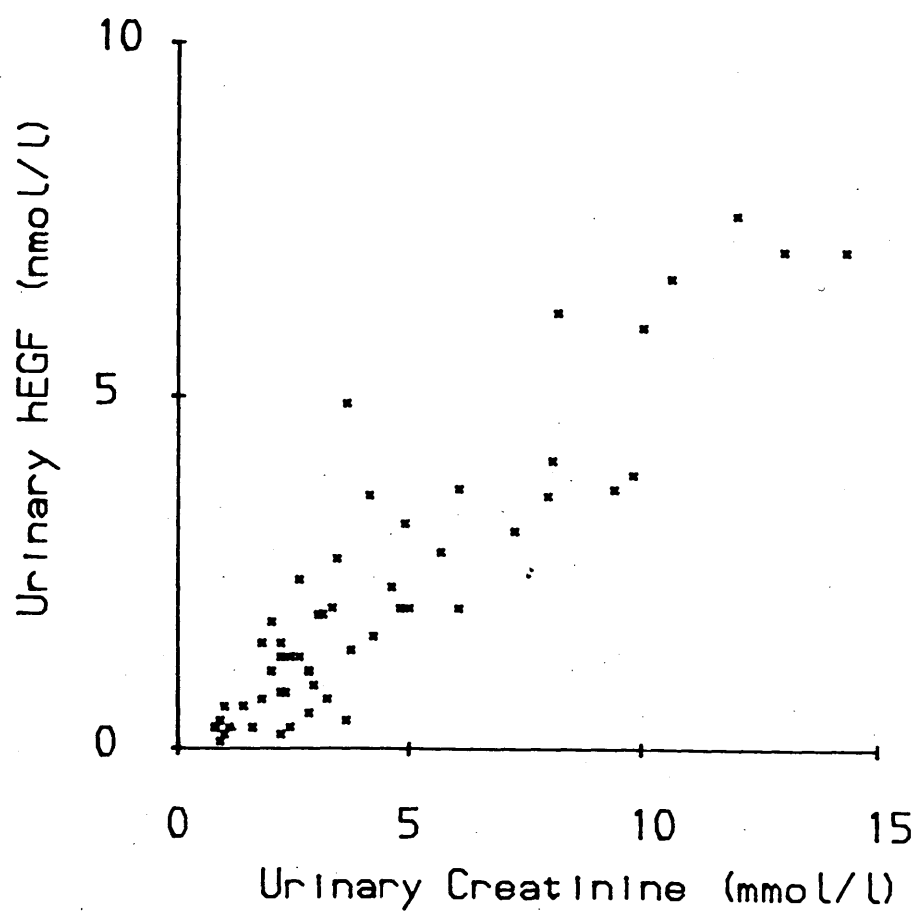


Figure 31 Epidermal growth factor : Relationship of hEGF to creatinine excretion in the diabetic population.

$r=0.91$, $p<0.001$

urine. Severe impairment of glomerular function will thus be necessary to cause appreciable changes in hEGF filtration. Although by definition, our microalbuminuric patients excreted only small quantities of albumin, they may nonetheless have considerable glomerular functional abnormalities, accounting for the demonstrated changes in EGF excretion. Creatinine is also filtered freely by the glomerulus. The strong correlation of urinary hEGF and creatinine concentrations shown here may reflect similar defects in glomerular function.

Previous work suggests that urinary hEGF is also of renal tubular origin. It may be that hEGF molecules destined for excretion are abnormally internalised into renal tissue, possibly under the influence of hyperinsulinaemia in some diabetic patients (115). Internalised hEGF degradation products may conceivably serve as a stimulus for the development of glomerulosclerosis. An alternative explanation is that the synthesis of hEGF is impaired in diabetic nephropathy. The role of the kidney in the metabolism of EGF is not completely understood. Studies performed in mice after surgical removal of the salivary glands led to the suggestion that mouse kidney may be a site of EGF synthesis(77). The kidney is also thought to be the source of EGF in rat urine(76). EGF can be visualised in the distal tubular cells by immunohistochemical techniques(116,117) and studies using mRNA probes for mouse prepro-EGF have localised synthesis of this precursor to the distal renal tubule(77). Harata and Orth(118) have assayed for hEGF in several human tissues and found the highest concentration in kidney homogenates. It could thus be speculated that mechanisms involving the synthesis or processing of hEGF from precursor molecules in the renal tubules might be impaired early in diabetic nephropathy, although it is generally thought that renal tubular function remains normal until very late in the disease process.

In the mouse, the release of stored EGF is stimulated by adrenergic agents(119). Autonomic neuropathy may thus be expected to influence the amount of EGF excreted in body fluids(120). However, none of the patients in this present study had clinical evidence of diabetic autonomic neuropathy.

In conclusion, an inverse relationship between albuminuria and urinary hEGF excretion in a population of diabetic patients with normal and declining renal function has been demonstrated. The reasons for this finding are not clear but the possible role of growth factors in nephropathy deserves closer scrutiny. However, our data do not support the hypothesis that the increased kidney size seen in early diabetic nephropathy is due to excessive amounts of EGF.

CHAPTER 9

Screening for early diabetic nephropathy : the value of the albumin:creatinine ratio

9.1 Aims

Collection of an overnight urine sample for measurement of AER is tedious for the patient and cumbersome for the laboratory. In addition, there may be errors in the collection or timing of the sample. The value of an albumin:creatinine ratio measured on an aliquot of the early morning urine has therefore been investigated.

9.2 Methods

Timed overnight urine samples were collected from 129 diabetic patients (67 insulin-dependent and 62 non-insulin dependent). Albumin concentration and creatinine were measured as described previously and AER ($\mu\text{g}/\text{min}$) and albumin:creatinine ratio (mg/mmol) calculated. The ability of an albumin concentration $>20 \mu\text{g}/\text{ml}$ and of albumin:creatinine ratios of >3.5 and >4.5 to predict "at risk" microalbuminuria (AER $30\text{--}150 \mu\text{g}/\text{min}$) was examined.

9.3 Results

The measured albumin concentration ranged from 0.38 to $212 \mu\text{g}/\text{ml}$ (median $30.5 \mu\text{g}/\text{ml}$) in the 129 urine samples. Forty six patients had AER values between 30 and $150 \mu\text{g}/\text{min}$ and thus were at risk of developing clinical nephropathy.

Using an albumin concentration $>20 \mu\text{g}/\text{ml}$ to predict an AER $>30 \mu\text{g}/\text{min}$, there were 22 false positives, giving a sensitivity of 91% and specificity 73.5%. Table 23 indicates the corresponding results using an albumin:creatinine ratio of >3.5 as an arbitrary cut-off. Sensitivity was 98% and specificity 69%. If the ratio is increased to 4.5, two patients would have given a false negative result, the sensitivity being 96% and specificity 80%.

9.4 Discussion

A suitable screening test, which may perhaps need to be repeated at regular intervals, must have a high sensitivity and specificity and be convenient for both patients and laboratory staff. The results presented above support those of Gatling et al(121) that simple measurement of albumin concentration in an aliquot of first morning urine is not sufficiently sensitive to use as a predictor of future diabetic nephropathy. An albumin:creatinine ratio >3.5 , measured on an aliquot of overnight urine, is a sensitive

Albumin concentration ($\mu\text{g/ml}$)		Albumin:creatinine ratio		Albumin:creatinine ratio		
Albumin excretion rate ($\mu\text{g/min}$)	≤ 20	≤ 3.5	> 3.5	≤ 4.5	> 4.5	
	≤ 30	61	22	26	66	17
> 30	4	42	1	45	2	44

Table 23. Relationship of AER to albumin concentration and albumin : creatinine ratio : cut-off points predicting AER $> 30\mu\text{g/min}$

predictor of AER $>30 \mu\text{g}/\text{min}$, but only at the expense of generating a large number of false positives, placing an extra load on patients and the laboratory. In a diabetic clinic caring for 500 insulin-dependent patients, 50 might be expected to have AER in the range of $30\text{--}150 \mu\text{g}/\text{min}$. Using an albumin:creatinine ratio cut-off of 3.5 in a first morning urine aliquot as initial screening, 190 patients would be recalled to provide a timed overnight sample. Of these, 49 would be true positives and one positive would be missed. If the cut-off was increased to 4.5, 140 timed overnight samples would be necessary, generating 48 true positives and 2 false negatives. The above population was selected to contain a large number of patients with high AER. The false positive rate may therefore be exaggerated. The price of the improved specificity with the 4.5 cut-off is decreasing sensitivity. For this reason, the most useful albumin:creatinine ratio cut-off is 3.5, although a larger study would confirm this. Although 38% of the patients would be asked to submit a timed overnight urine sample, compliance would be high, in the knowledge that they might be at risk of developing renal disease.

Our uncertainty of the time-course of development of diabetic nephropathy and the day-to-day variation in albumin excretion in individual diabetic patients(105,111) suggest that it may be necessary to screen diabetic subjects at regular intervals, perhaps once every six months, until persistent microalbuminuria develops. It is thus especially important that the test used is acceptable to patient and laboratory. An aliquot of the first morning urine passed is simple and convenient for the patient to provide and easy for the laboratory to handle. Many patients already bring such a specimen to the diabetic clinic. Urine creatinine concentrations can be quickly measured by the autoanalyser and in an average-sized diabetic clinic no more than one albumin assay would be required per week.

I therefore suggest that an albumin:creatinine ratio measured in an aliquot of the first morning urine would provide an initial screening test for those at risk of developing clinical diabetic nephropathy. Formal measurement of AER is then necessary on all those screening positive to identify those with microalbuminuria.

CHAPTER 10

Evaluation of rapid techniques for albumin measurement

10.1 Aims

It seems likely that the detection and monitoring of low levels of albuminuria will become a routine requirement in the diabetic clinic. Simple, reproducible but accurate methods are required to allow the throughput of large numbers of samples. A new reagent tablet, Microbumintest (Ames Limited, Stoke Poges, Slough, UK) for detection of urine albumin concentrations $>40 \mu\text{g/ml}$, and a commercial kit using an automated immunoturbidimetric method (Urin-Pak, Ames Limited, Stoke Poges, Slough, UK) have been evaluated.

10.2 Microbumintest

(a) Chemical principle

Microbumintest reagent tablets consist of a cellulose base impregnated with bromophenol blue and salicylic acid. One drop of urine is placed on the tablet, protein in the sample being adsorbed to the tablet surface. Following the addition of 2 drops of water, the urine is washed through the tablet but the protein remains on the surface where it reacts with the indicator bromophenol blue to produce a bluish-green colour. Results are compared with the colour chart provided by the manufacturers as:

1. negative - no colour change
2. borderline - weak colour change
3. positive - strong colour change

The manufacturers claim that samples classified 2 or 3 have an albumin concentration $>40 \mu\text{g/ml}$.

(b) Methods

Urine samples were obtained from 106 diabetic patients. There were 54 overnight collections and 52 early morning urine samples (EMU). All were Albustix negative. Each sample was tested by one observer in batches of 12. Twenty-four samples were tested independently by 8 observers on 2 occasions. One low ($5.2 \mu\text{g/ml}$) and one high ($150 \mu\text{g/ml}$) reference samples were tested as quality controls in each batch. The albumin concentration in each urine sample was also determined by radioimmunoassay.

(c) Results

The mean albumin concentration was 34.5 (range 1.5-149) $\mu\text{g}/\text{min}$ in all samples, 36.1 (1.5-149) in the overnight collections and 33.0 (2.5-145) in the EMU samples.

The Microbumintest categorisation of these 106 samples by the single observer is shown in Table 24 and Figure 32. If only category 3 results were considered to indicate an albumin concentration $>40 \mu\text{g}/\text{ml}$, the sensitivity of the test was 30% and specificity 98%. When both categories 2 and 3 were considered positive, the sensitivity was 84% and specificity 80%.

When 8 operators tested 24 urine samples, only 81 out of 192 (42%) samples were correctly classified. There was complete agreement by all 8 testers in only 8 samples, by 7 testers in 2 samples and 6 testers in 3 samples. When each operator assayed each sample twice, there were 37 out of 192 (19%) changes of category, with a mean number of changes per tester of 4.5 (range 1-8).

(d) Discussion

The very high false negative rate and the large inter- and intra-operator variability make Microbumintest unsuitable for routine screening for microalbuminuria. The results are reminiscent of the low sensitivity and lack of precision seen with the use of Albustix in a clinic situation(122). One explanation for the low specificity and sensitivity may be that Microbumintest is not specific for albumin, but may also react with other urinary proteins present in variable amounts in different urine samples.

10.3 Urin-Pak

(a) Assay principle

This immunoturbidimetric kit uses goat anti-human albumin antibody and precipitation by 40g/l polyethylene glycol in 50 mmol/l Tris buffer, pH7.0. The standard solutions are prepared from human albumin in physiological saline. The analysis is performed on the automatic Cobas Bio centrifugal analyser (Roche UK, Welwyn Garden City, Herts, UK). The programmable settings for the instrument are shown in Table 25.

(b) Methods

The 106 urine samples used in the evaluation of Microbumintest, described above, were also assayed by reference radioimmunoassay and Urin-Pak.

Albumin Concentration ($\mu\text{g/ml}$)	Microbumintest Category		
	1	2	3
≤ 40	45	10	1
> 40	8	27	15

Table 24 Categorisation of 106 urine samples by Microbumintest.

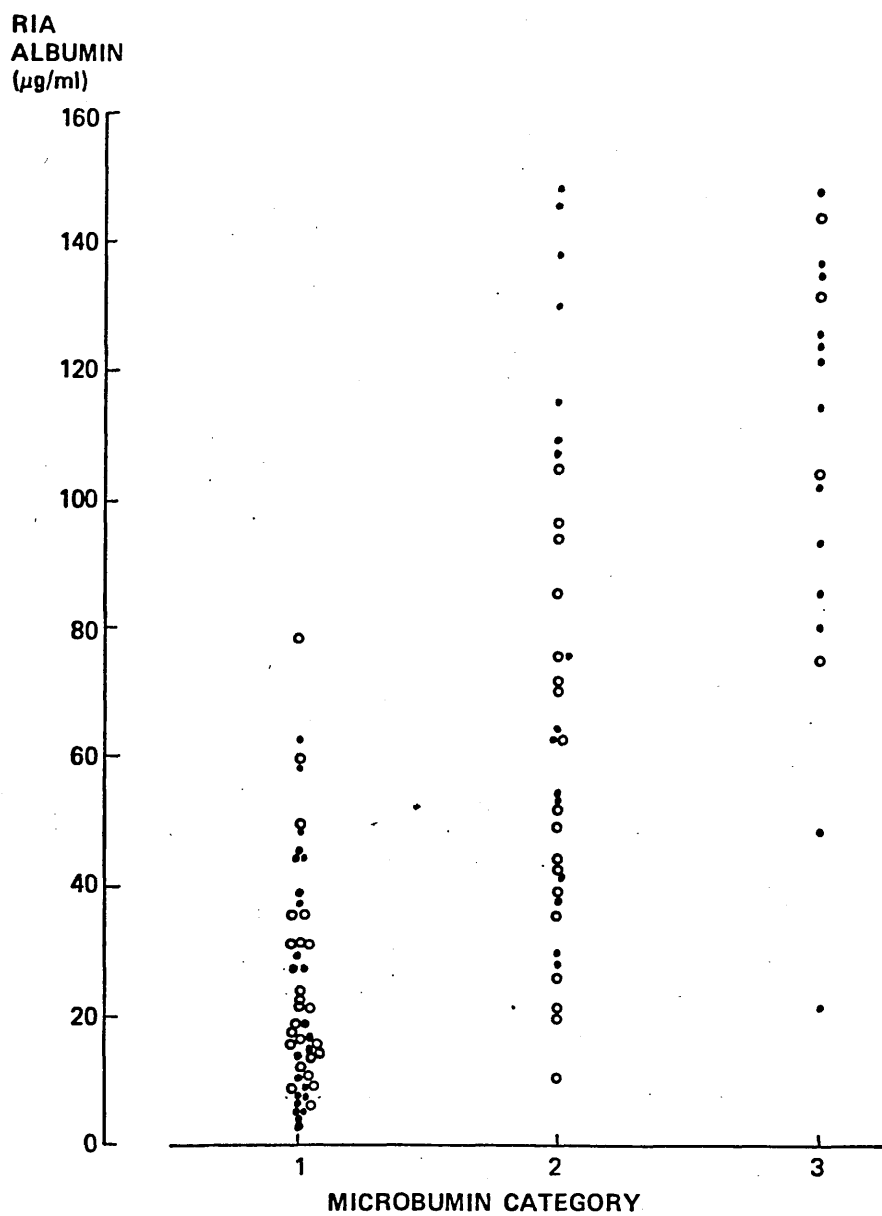


Figure 32 Categorisation of 106 urine samples by Microbumintest

● overnight urine sample

○ early morning urine sample

Standard curve	0 - 160 $\mu\text{g/ml}$
Temperature	25°C
Wavelength	340 nm
Sample volume	25 μl
Reaction time	300 sec

Table 25 Evaluation of Urin-Pak: Programmable variables for centrifugal analyser.

Four samples, concentration range 5.2-155.0 µg/ml, were assayed six times in each assay for determination of the intra-assay coefficient of variation and also in each of 6 assays for determination of the inter-assay CV. Six solutions of known albumin concentration (range 5-160 µg/ml) were prepared in diluent buffer for estimation of recoveries. The stability of diluted standards was checked by storing prepared standards at 4°C for 16 days. Fourteen samples, albumin concentration range 5.2-151.2 µg/ml, were assayed using the stored standards at intervals from days 0-16.

(c) Statistical methods

Comparison of the methods was by Pearson correlation coefficient. Assay variability was assessed by the coefficient of variation.

(d) Results

Albumin concentrations as measured by the reference method are given in Table 26. The correlation of the two methods is shown in Figure 33. In twelve samples, the Urin-Pak albumin concentration was <5 µg/ml and in eight >160 µg/ml. After exclusion of these 20 samples, the correlation coefficient was 0.91 ($p < 0.001$) with a regression equation of:-

$$\log \text{albumin}_{\text{Urin-Pak}} = 1.02 \log \text{albumin}_{\text{RIA}} - 0.078$$

Correlation coefficients for the overnight and EMU samples were similar ($r=0.92$ and $r=0.90$ respectively). However, 24 of the 86 samples (28%) lay outside $\pm 20\%$ of the line of identity (Figure 33). On repeat assay of these samples, the mean percent change in the albumin concentration was 1.1% (range -56 - +44) by reference method and -4.8 (range -68 - +33) by Urin-Pak and all remained outside $\pm 20\%$ of the line of identity.

The intra and inter assay coefficients of variation are shown in Table 26. These were 6.2% or less at all concentrations. The mean recovery was 96.5% (range 89.7 - 100.3). The change in albumin concentrations in samples measured by stored standard solutions over a 16 day interval is shown in Figure 34. The mean percent change in the albumin concentrations from day 0 to 16 was -5.1% (range -23.9 - +5.1).

URIN-PAK
ALBUMIN
CONCENTRATION
($\mu\text{g/ml}$)

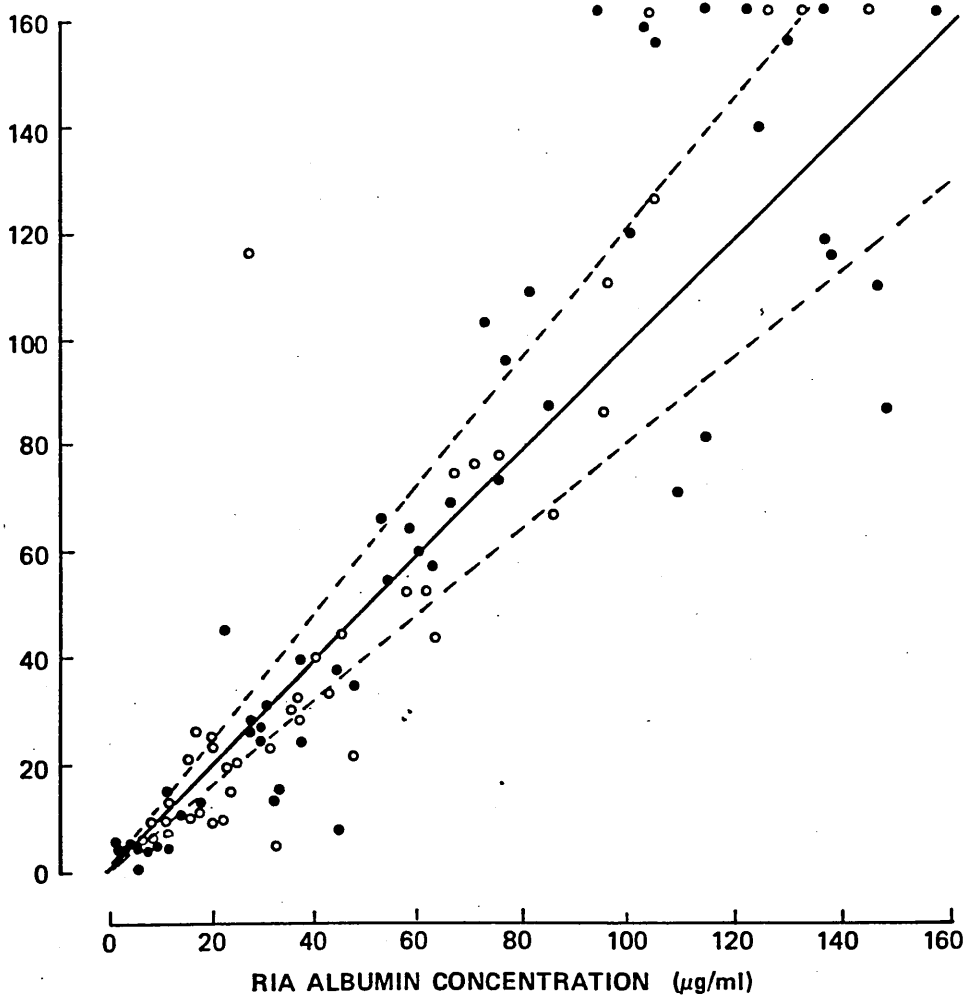


Figure 33 Comparison of albumin measurements in overnight (●) and early morning urine samples (○) by Urin-Pak and radioimmunosassy.

— $y = x$

- - - $\pm 20\%$ of line of identity

$r=0.91$

Albumin Concentration ($\mu\text{g/ml}$)	Coefficient of Variation (%)	
	Intra-assay	Inter-assay
5.6	2.3	5.1
12.5	1.3	6.2
40.7	5.2	5.2
155.0	3.0	3.2

Table 26 Evaluation of Urin-Pak: intra and inter-assay coefficient of variation at 4 albumin concentrations

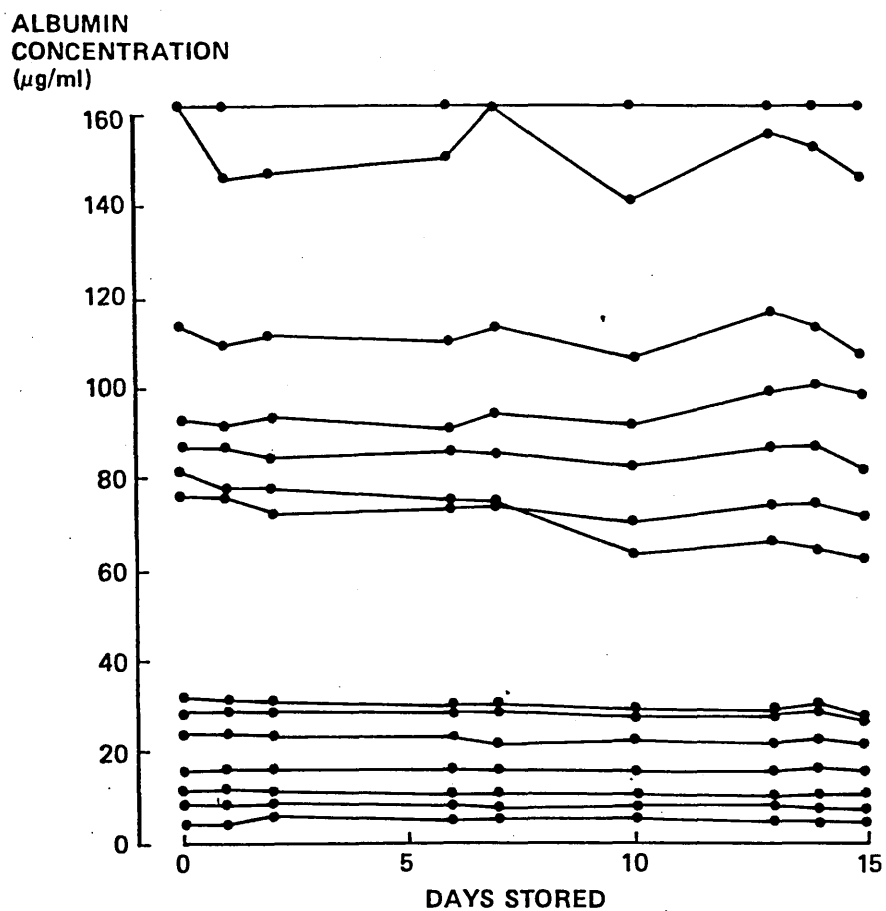


Figure 34 Evaluation of Urin-Pak: effect of storage of diluted standard solutions for up to 16 days.

(e) Discussion

Urin-Pak proved to be a rapid and easy to use method for measurement of urine albumin. Although the overall correlation with the reference method was acceptable, the number of samples outside $\pm 20\%$ of the line of identity is worrying. The low percentage change on repeat assay of these samples by both methods suggests a true assay difference rather than an analytical error. It may be that some additional factor such as urine pH or solute concentration is exerting a consistent effect in one or other assay.

Standard solutions appear stable when stored diluted at 4°C for at least 16 days, reducing reagent wastage and assay cost. Recoveries were acceptable and assay variation small. Overnight and EMU samples behaved similarly in the assay.

In conclusion, Urin-Pak is a quick, easy to use and precise method for the determination of low concentrations of urine albumin. However the consistent difference in albumin concentration in some samples in the new and reference method requires further investigation.

CHAPTER 11

Conclusions

11.1 Prevalence of abnormal albumin excretion

In 106 healthy adults, the upper limit of normal albumin excretion rate in a timed overnight urine collection was 10.5 $\mu\text{g}/\text{min}$. In an unselected out-patient clinic population, 22.3% of IDDM patients and 33.6% of NIDDM patients had excretion rates outwith the normal range. Seven percent of IDDM subjects had AER 30-150 $\mu\text{g}/\text{min}$ and 5% >150 $\mu\text{g}/\text{min}$; the corresponding figures for NIDDM were 10% and 6%. The prevalence of microalbuminuria in this population agrees well with other recently published English studies(97,123) but is less than in several Scandinavian studies(47,95,96,124,125). Similarly, the prevalence of AER >150 $\mu\text{g}/\text{min}$ is also less than recorded in other recent series(51,126). Some of this variation is undoubtedly due to differences in methodology and some may be due to real differences between populations: Asian(127), Indian(128), Afro-Caribbean(99) and American black people(129) may be at higher risk of diabetic nephropathy. However, the true incidence of diabetic nephropathy, at least in IDDM, may also be declining(8,21,130).

The belief that end-stage renal disease is less common in NIDDM than IDDM patients has been challenged recently by evidence from studies of Pima Indians. After 20 years duration of diabetes, this group has a cumulative incidence of 40.8 cases per 1000 person-years(131), a rate similar to insulin-dependent patients of similar diabetes duration followed at the Joslin clinic(21). Duration of diabetes was a strong risk factor for ESRD, suggesting that the much higher rate of ESRD compared with other NIDDM groups may be because diabetes occurs at an earlier age in Pima Indians. An alternative explanation is that Pima Indians carry a particular susceptibility to diabetic nephropathy as well as to diabetes. In keeping with the high incidence of ESRF, there is also a high incidence of proteinuria, also as high as in IDDM patients(132).

11.2 The associated features of abnormal albumin excretion

In IDDM, abnormal albumin excretion is associated with male sex and the presence of retinopathy, whilst in NIDDM the concomitant features are higher systolic blood pressure, retinopathy and peripheral vascular disease. A preponderance of males has long been a recognised feature of nephropathy and a recent study has shown an excess of males with microalbuminuria(123). Similarly, whilst retinopathy has been well-recognised as a common, perhaps even an obligatory, feature of end-stage renal

disease, it is now accepted also as a feature of microalbuminuria(96,133). Given that persistent proteinuria in NIDDM has recently been shown to carry the same risk of increased mortality from renal and cardiovascular disease as in IDDM(134) it is perhaps not surprising that in this survey, abnormal albumin excretion was associated with peripheral vascular disease and higher blood pressure. A recent study has shown a significant association between albumin excretion and coronary artery disease in NIDDM(135). However, in a 15 year follow-up of non-diabetic and diabetic men in the Whitehall study, although diabetic men carried a much higher risk of cardiovascular mortality, there was no significant relationship between the relative risk and duration of diabetes(136). This prompted the authors to suggest an associating link, perhaps genetic, between the two conditions, rather than a causal relationship.

The value of microalbuminuria and macroproteinuria as predictors of cardiovascular mortality in IDDM(137) and NIDDM(133) have both been further emphasised recently. Why should diabetic nephropathy and generalised cardiovascular disease be associated? In 1953, Lundbaek suggested that they were alternative expressions of a generalised specific diabetic angiopathy(1). Albuminuria may simply represent a generalised increase in the transcapillary escape rate of albumin, shown to be elevated in IDDM patients with microangiopathy and with hypertension but no microangiopathy(138). The transcapillary escape rate is increased in IDDM patients with micro- and macroproteinuria(139) and can be altered by changes in diabetic control(140). An increased passage of albumin and possibly other molecules into arterial walls may increase the atherogenic process(141).

One group has hypothesised that inheritance of abnormal enzymes in the extracellular matrix, coupled to the presence of diabetes, is responsible for both albuminuria and widespread vascular damage(142)

An alternative explanation of the association is of blood lipid disturbances. Blood lipid levels are elevated in end-stage renal disease(143). However, recent studies on IDDM patients with persistent proteinuria but not renal failure, have shown abnormalities in lipid levels compared to matched normoalbuminuric populations(144,145,146) and two studies have in addition shown increased lipid levels in the microalbuminuric range (146,147). In a study of carefully matched patients with and without nephropathy, the only differences found were higher blood pressure and serum cholesterol in those with nephropathy(148).

It is noteworthy that the Framingham study revealed an increased mortality in subjects with proteinuria, although numbers were too small to study separately those without diabetes, hypertension or heart failure(149). Recently, microalbuminuria in non-diabetic subjects has been shown to be associated with coronary heart disease and peripheral vascular disease(150).

11.3 Variation in albumin excretion

This study has demonstrated a large day-to-day variation in albumin excretion in normal subjects and diabetic patients with all degrees of albuminuria. Several studies have recently confirmed this in normal children(151) and normal and diabetic adults(152,153) and one has suggested that the variance is greater in diabetic subjects(153). Calculation of the albumin:creatinine ratio did not reduce the variability of the overnight collection(152).

A great variation in rate of change over a longer period of time in both normoalbuminuric and microalbuminuric patients has been demonstrated here. Several recently published studies have shown a rather slow overall rate of change in albumin excretion, some patients remaining at their baseline level, some fluctuating between categories and only a few exhibiting steady progression(111,154,155). This marked day-to-day variation and longer-term variability implies that no patient should be categorised on the basis of one measurement of albumin excretion. In intervention trials, adequate controls must be included since no assumptions about the rate of change of albumin excretion can be made.

11.4 Bromhexine and albumin excretion

No effect of one month's treatment with bromhexine on albumin excretion has been demonstrated. It remains to be seen whether longer therapy will be effective. Glycoprotein accumulated in the basement membrane and mesangium is not static. In experimental animals, correction of the diabetic state by islet cell transplantation or insulin treatment results in improvement of the basement membrane and mesangial lesions(156,157).

11.5 Screening for microalbuminuria

Measurement of the albumin:creatinine ratio in an early morning sample of urine has been shown to be a good predictor of an elevated AER in a timed overnight urine

sample. In children, an albumin concentration $>20 \mu\text{g/ml}$ in a first morning urine sample has been shown to correlate well with 24h urinary albumin excretion(158). Using an albumin:creatinine ratio did not improve the relationship. In adults, a further study has shown that both a timed overnight and morning albumin excretion rate have high specificity and sensitivity in predicting a 24h excretion rate of 20-200 $\mu\text{g/min}$ (159). The sensitivity and specificity were further improved by use of an AER:creatinine ratio.

It thus seems that a timed collection may not be necessary as an initial screening test for microalbuminuria. Measurement of the albumin:excretion ratio in an early morning urine sample may have sufficient sensitivity and specificity.

There may be occasions on which a semi-quantitative screening test is deemed suitable. Microbumintest, evaluated here, is not suitable because of its poor discrimination and large intra- and inter-operator variability. The low specificity of this test has recently been confirmed(160). The latex bead immunoagglutination tests recently described appear to be easy to use, specific for human albumin and have high sensitivity(161,162,163).

11.6 The future

Long-term natural history studies examining the rate of progression from normo- to microalbuminuria and from micro- to macroproteinuria are required, along with detailed clinical studies in an effort to try to categorise those patients in whom microalbuminuria will develop. Thereafter, ways of decreasing the excess risk of cardiovascular and renal disease carried by patients with microalbuminuria should be explored. The main avenues appear to be control of blood pressure and blood glucose, and reduced dietary protein intake.

(a) Blood pressure

In this study, systolic blood pressure was related in multiple regression analysis to AER in NIDDM, and in univariate analysis in IDDM. Other studies have shown elevated blood pressure in IDDM patients with microalbuminuria(37,47,164). It is noteworthy that these patients do not have hypertension by any formal definition. Only when matched with an appropriate peer-group is the elevated blood pressure obvious(165). Antihypertensive therapy reduces microalbuminuria acutely(166) and a carefully

controlled trial has shown sustained reduction in albuminuria for 1 year(167,168). An uncontrolled trial has shown sustained reduction in albumin excretion and a slight fall in GFR over 5 years(169). A long-term, randomised, properly controlled trial is needed. Additionally, antihypertensive therapy reduced the transcapillary escape rate of albumin both acutely(170) and in the long-term(171) and thus may reduce the risk also of vascular disease.

It has been suggested that angiotension-converting enzyme inhibitors may offer an advantage over other antihypertensive therapy by selectively reducing intra-glomerular pressure in macroproteinuric(172,173,174) and microproteinuric patients(167). A comparison of antihypertensive regimens in micro- and macroalbuminuria is required.

A short-term study comparing the effects of an angiotensin converting enzyme inhibitor and a calcium antagonist found similar hypotensive action without alteration in renal blood flow, GFR and filtration fraction(175). The albumin excretion rate and fractional clearance of albumin were reduced to a similar extent by both drugs.

Animal studies have demonstrated that the angiotensin converting enzyme inhibitor enalapril retards the development of glomerular basement membrane thickening and reduces albumin excretion in diabetic, spontaneously hypertensive and diabetic and hypertensive rats(176).

The generally accepted view until recently was that hypertension was a consequence of rather than cause of diabetic nephropathy. However, the demonstration of elevated blood pressures very early in the course of the disease challenged this assumption. Viberti studied parents of IDDM patients with and without nephropathy(177). Blood pressure was higher in the parents of the patients with nephropathy. They further found that the rates of sodium-lithium countertransport in red blood cells, a marker for essential hypertension and whose activity is genetically determined(178) were higher in the group with nephropathy(179). These observations have been confirmed by another group(180) and a third group has demonstrated that diabetic nephropathy occurs in familial clusters(181). It thus seems possible that those diabetic patients at risk of nephropathy have inherited the predisposition to hypertension which may promote the development of diabetic nephropathy.

(b) Blood Glucose Control

One randomised, controlled trial has demonstrated that albumin excretion was unchanged after 1 year of strict blood glucose control(182) but after 2 years, no patient in the strictly-controlled group had progressed to clinical nephropathy whereas 5 out of 18 in the conventionally controlled group had progressed(183). Additionally, the fractional clearance of albumin had increased in the conventionally controlled group but not in the very well controlled group. However, this study included a substantial number of patients who although they had elevated albumin excretion, had not been shown to be at risk of progression. Also the numbers studied were rather small. A further trial is therefore required.

A comparison of renal histological changes in diabetic patients at least 1.9 years after renal transplantation with or without pancreas transplantation, showed that the recipients of pancreas transplants had no evidence of structural diabetic disease and had small glomerular volumes and less mesangial expansion compared to those receiving only renal transplants(184). This evidence suggests that good blood glucose control, if started sufficiently early in the course of diabetes, can prevent the development of diabetic nephropathy.

(c) Dietary Protein Restriction

Reduction of dietary protein intake to a moderate intake of 40g per day reduces albumin excretion(185) and may slow the rate of decline in glomerular function(38,186,187) in IDDM patients with overt nephropathy. In patients with microalbuminuria, a 3 week trial of a reduced protein diet has shown reduction in albumin excretion and in GFR from hyperfiltration levels(188). Obviously, larger and longer-term studies are required in both persistent and microalbuminuria although the difficulties of adding protein restriction to an already limited diet are enormous.

(d) Other measures

Restoration of blood lipids to more normal levels, along with measures to improve blood pressure and blood glucose control, may help to reduce the high cardiovascular mortality in both macro- and microproteinuria. Abnormal prostaglandin production by the mesangium and impaired mesangial contractility to prostaglandin may have a role in the pathogenesis of glomerular hyperfiltration in nephropathy(189). One trial of a specific thromboxane inhibitor resulted in a decrease in albumin excretion in IDDM patients with microalbuminuria(190). Further work is required.

The enzyme aldose reductase is the rate-limiting enzyme in the polyol pathway which converts glucose to its corresponding sugar alcohol sorbitol. Sorbitol accumulates in the glomeruli of streptozotocin diabetic rats(191) and may, by osmotic disruption or depletion of myo-inositol, be involved in the pathogenesis of diabetic nephropathy. The activity of aldose reductase has recently been shown to be increased in kidneys from diabetic rats(192). Inhibition of aldose reductase by sorbinil reduces glomerular basement membrane thickening and mesangial expansion without affecting albumin excretion or renal functional parameters in experimental diabetes(193). However, a further study using a different aldose reductase inhibitor, statil, found no effect on histological or functional parameters(194). The reasons for such discrepancies are not clear but may have resulted from differences in the inhibitor used, different duration and severity of diabetes and different methods of histological assessment. The use of aldose reductase inhibitors in the various stages of human diabetes obviously needs to be explored. A very recent study has suggested that aldose reductase inhibition may reduce albumin excretion in IDDM patients with microalbuminuria(195).

A large Danish study comparing IDDM patients who had died within 35 years of diagnosis of diabetes with patients who had survived for more than 40 years, found that the early deaths were characterised by male preponderance, poor metabolic control and less frequent attendance at a specialised diabetes unit(196,197). Apart obviously from male sex, these factors are open to intervention. Hopefully by attacking these factors, along with attention to the other factors described above, it will be possible to reduce the excess mortality from renal and cardiovascular disease in diabetic patients.

11.7 Unifying Hypothesis

In 1953 Lundbaek suggested that the long-term manifestations of diabetes were different expressions of one generalised diabetic vascular disease(1). Reaven has recently proposed a "syndrome X" of resistance to insulin-stimulated glucose uptake and hyperinsulinaemia as a common underlying feature of NIDDM, hypertension and coronary artery disease(198). The elevated rates of sodium-lithium countertransport described in nephropathic diabetic patients(179,180) may provide a clue to the mechanisms underlying these syndromes. The sodium-lithium countertransport system is thought to be one mode of operation of the sodium-hydrogen antiport, which amongst other functions, regulates cell growth and sodium reabsorption by the kidney(199). It has recently been shown that fibroblasts from nephropathic diabetic

patients have higher sodium-hydrogen transport activity and more cell growth than cells from non-nephropathic patients(200). Proximal renal tubular absorption of sodium is increased in hypertensive NIDDM patients and is associated with fasting hyperinsulinaemia(201). thus overactivity of the sodium-hydrogen antiport could explain the renal hypertrophy and renal and systemic hypertension of diabetes and could eventually lead to renal impairment. Overactivity of the sodium-lithium countertransport system is also associated with lipid abnormalities in the general population(202). Hyperlipidaemia may further promote renal damage(203) and may, in combination with systemic hypertension and the generalised increase in vascular permeability observed in diabetes(142), accelerate cardiovascular disease.

It is possible that insulin resistance may play a role in the mechanism linking increased sodium-lithium countertransport, hypertension and hyperlipidaemia(204). Insulin resistance is associated with essential hypertension(205) and coronary heart disease(206) in the non-diabetic population. It has recently been demonstrated that in hypertensive, non-proteinuric IDDM patients, increased activity of the sodium-hydrogen antiport is associated with microalbuminuria, left ventricular hypertrophy and increased insulin resistance(207).

Thus the combined triggers of diabetes (insulin resistance) and increased activity of the sodium-hydrogen countertransport system may initiate a vicious, self-fuelling cycle of cell overgrowth and hyperfunction, renal and systemic hypertension, and lipid abnormalities which eventually culminate in the widespread vascular and renal disease seen in long-standing diabetes mellitus.

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**THE EFFECT OF BROMHEXINE ON ALBUMIN EXCRETION
IN INSULIN DEPENDENT DIABETES**

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SUMMARY

The effect of the mucolytic agent bromhexine, 72 mg daily for one month, on albumin excretion in insulin dependent diabetes was investigated in a double-blind, randomised, placebo-controlled study. Nine patients with normal albumin excretion [overnight albumin excretion rate 3.2 (2.1-8.8) $\mu\text{g min}^{-1}$; mean (range)], six with microalbuminuria [36 (22-95) $\mu\text{g min}^{-1}$] and six with macroalbuminuria [321 (201-1215) $\mu\text{g min}^{-1}$] participated. Albumin excretion was similar after treatment with bromhexine and placebo in all 3 groups [normoalbuminurics 3.6 (1.7-13.5) versus 3.3 (1.9-13.2) $\mu\text{g min}^{-1}$; microalbuminurics 40 (20-128) versus 37 (20-103); macroalbuminurics 396 (247-2160) versus 443 (292-2592)]. Excretion of β_2 -microglobulin and plasma creatinine were identical at the end of each treatment. Blood glucose control and blood pressure remained constant throughout the study in the 3 groups. We conclude that bromhexine 72 mg daily for 1 month had no effect on albumin excretion in IDDM patients with normal and pathological albuminuria.

Key words: Bromhexine, albumin excretion, IDDM, diabetic nephropathy

INTRODUCTION

The development of nephropathy in insulin dependent diabetes (IDDM) is marked by increasing urinary albumin excretion. Clinical nephropathy is defined as the development of persistent Albustix-positive (Ames Ltd, Stoke Poges, Slough, UK) proteinuria (1). Prior to the appearance of persistent proteinuria, there is a stage of increased albumin excretion above normal but short of Albustix-positive proteinuria. Several studies have shown that IDDM patients exhibiting this so-called "microalbuminuria, later develop persistent proteinuria (2-5). The phase of persistent proteinuria has proved irreversible, antihypertensive therapy simply slows the rate of decline of glomerular filtration rather than halting the deterioration(6). It is hoped however, that treatment of the earlier stage of microalbuminuria may be more fruitful. Improved blood glucose control may prevent the progression to persistent proteinuria(7) and reduction in dietary protein has been shown to reduce albumin excretion, at least in the short-term(8).

The histological features of diabetic nephropathy are of increasing deposition of glycoprotein and oligosaccharide material in the basement membrane and mesangium(1). Accumulation of such material in the glomerular tuft may reduce the glomerular filtration surface(9) whilst qualitative abnormalities of the oligosaccharide material may lead to loss of anionic charge on the basement membrane and enhanced passage of albumin(10). The vinka alkaloid bromhexine is a mucolytic agent thought to alter glycoprotein synthesis. Bromhexine has been shown to decrease the urinary excretion of carbohydrate containing substances in IDDM(11) and to decrease glomerular volume in experimental diabetes(12) and this is of potential benefit in the treatment of diabetic nephropathy.

We have therefore examined the effect of bromhexine on albumin excretion in IDDM patients with varying degrees of nephropathy.

METHODS

Subjects

Twenty one IDDM patients were studied. They were divided into 3 groups on the basis of overnight albumin excretion rates in 2 out of 3 sterile, ketone-free urine collections:

Group 1: normoalbuminurics, AER $\leq 10.5 \mu\text{g min}^{-1}$

Group 2: microalbuminurics, AER 10.6–150 $\mu\text{g min}^{-1}$

Group 3: macroalbuminurics, AER $>150 \mu\text{g min}^{-1}$

Patient characteristics at baseline are shown in tables 1 and 2. One patient in group 3 took carbamazepine for painful peripheral neuropathy and thyroxine for auto-immune thyroid disease, in constant dosage throughout the study. No other subject took any medication other than insulin throughout the study and diet was unaltered.

Study design

The study was a placebo-controlled, double-blind randomised trial. Subjects were randomised at baseline, within their albumin excretion groups, to receive bromhexine 8 mg or placebo tablets, three tablets three times a day each for 4 weeks. There was a 2 week "wash-out" period between each treatment period when all patients took placebo tablets. Clinical and laboratory measurements were made at the beginning and end of each treatment period. Patients collected 3 timed overnight urine samples for measurement of albumin, β_2 -microglobulin at each time point. Blood was drawn for determination of HbA_{1c}, serum β_2 -microglobulin and fructosamine and plasma creatinine. Blood pressure was measured in the sitting position after 5 minutes rest, using Korotckoff phase V diastolic pressure. A tablet count was performed to check compliance. On the day prior to each clinic visit, patients performed 4 capillary blood glucose tests using BM Test Glycemie 1-44 strips (Boehringer, Mannheim, West Germany), one test before each main meal and one before bed. The test strips were returned to an air-tight container and brought to hospital next day, where they were read by reflectance meter. The study was approved by the local ethical committee and all patients gave informed consent.

Laboratory analysis

Urine was collected into 0.5ml 4M NaOH and 0.5ml 0.1% merthiolate. Albumin was measured by single-antibody radioimmunoassay(13). The inter-assay coefficient of variation (CV) was 4.1% at $3.5 \mu\text{g ml}^{-1}$ and 10.0% at $125 \mu\text{g ml}^{-1}$. The upper limit of normal in our laboratory for overnight albumin excretion rate is $<10.5 \mu\text{g min}^{-1}$. Urinary and serum β_2 -microglobulin were measured by radioimmunoassay(14). The inter-assay CV was 4.7% at $77.3 \mu\text{g l}^{-1}$ for urine and 7.0% at 1.63 mg l^{-1} in serum. Glycosylated haemoglobin was measured by Corning electrophoresis, the normal range being 5.0-7.5%. Fructosamine was assayed by a modification of the method of Johnson et al using a Cobas Bio centrifugal analyser(15). The reference range is 1.79-2.35 mmol fructosamine 100g albumin^{-1} .

Plasma creatinine was determined by modified automated Jaffe technique. Excretion rates for albumin and β_2 -microglobulin were calculated from the respective urinary concentrations and the duration and volume of the overnight urine collection.

Statistical analysis

Results were analysed at the end of each treatment period, irrespective of order and also at each time point, irrespective of treatment, to look for an order effect. The mean of the 3 albumin and β_2 -microglobulin excretion rates at each time point was calculated to allow for day-to-day variation. Albumin and β_2 -microglobulin excretion rates were log transformed before analysis to correct for skewed distribution. The differences within and between groups was assessed by paired or unpaired Student's t-test, with correction for multiple testing. Results are expressed as mean (range).

RESULTS

Albumin excretion rates were similar after 1 month's treatment with bromhexine or placebo in all 3 groups [group 1: 3.6(1.7-13.5) versus 3.3(1.9-13.2); group 2: 40(21-129) versus 37.1(20-103); group 3: 397(248-2160) versus 443.4(292-2593) $\mu\text{g min}^{-1}$; bromhexine versus placebo, Table 3 and Figure 1]. Similarly, other measures of renal function were not different between the treatment periods in each group. (Table 3). There was no change in systolic blood pressures or blood glucose control as judged by HbA_{1c} , fructosamine or laboratory read, self-collected capillary blood glucose concentrations (Table 4 and Figure 2) throughout the study. A tablet count revealed that patients had taken 97% bromhexine and 98% placebo tablets. No adverse effects of treatment were noted

DISCUSSION

We have demonstrated no effect of treatment with bromhexine 72 mg daily for one month on albumin excretion or any other measure of glomerular or renal tubular function in IDDM patients with varying degrees of albuminuria. Nor was there any change in blood glucose control or blood pressure, variables which by themselves alter albumin excretion. The hallmark of diabetic nephropathy is accumulation of abnormal glycoproteins in the basement membrane and mesangium(16), reducing the glomerular filtration surface, with a strong correlation of glomerular filtration rate and filtration surface for nephron(9). It might be expected that bromhexine, by its mucolytic action on glycoprotein metabolism, might reduce glycoprotein deposition in the glomerulus, increasing the available filtration surface and improving kidney function. Previous work has shown a decrease in the excretion of carbohydrate-containing materials in diabetic patients after one months therapy with an identical dose of bromhexine(11). However, albumin excretion was not measured and the patients may all have had normal renal function. It may be that albumin excretion elevated above normal represents irreversible structural damage in IDDM, suggesting that glycolytic agents would only be effective as primary prevention, rather than in the breakdown of already-deposited glycoprotein. It may be that a longer treatment period is required to show changes in albumin as opposed to carbohydrate excretion, or that a higher dose of bromhexine is required. It has been suggested that the major determinant of albumin excretion in IDDM is the loss of anionic change on the basement membrane, perhaps due to loss of heparan-sulphateproteoglycan, rather

than actual accumulation of glycoprotein(10). If bromhexine was ineffectual in increasing anionic charge on the basement membrane, no effect would be seen on albumin excretion.

In experimental diabetes, bromhexine has been shown to significantly decrease glomerular volume, with a tendency to reduce basement membrane thickening and kidney volume(12). However, only in those animals given a large dose (25 mg kg^{-1}) bromhexine were significant changes seen.

In conclusion, we have shown no effect on albumin excretion of one months treatment with bromhexine 72 mg daily in IDDM patients with varying degrees of albuminuria.

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LEGENDS

Figure 1. Albumin excretion rate in 21 IDDM patients with varying albumin excretion at the end of one months treatment with bromhexine and placebo.

Figure 2. Laboratory read, self-collected capillary blood glucose concentrations in IDDM patients with normoalbuminuria (lower panel), microalbuminuria (middle panel) and macroalbuminuria (upper panel) after one months treatment with bromhexine (●—●) or placebo (○---○).

TABLE 1. Patient clinical characteristics at baseline.

	GROUP		
	1	2	3
n	9	6	6
Age	34.7	47.2**	33.0 ¹¹
(years)	(23-46)	(37-54)	(21-46)
Duration Diabetes	15.9	22.8	23.5
(years)	(6-29)	(7-32)	(19-31)
Retinopathy	3:0	4:2	3:3
(Background : Proliferative)			
HbA _{1c}	8.1	10.6*	10.2 ²
(%)	(6.6-10.7)	(8.1-13.1)	(8.6-11.7)
Fructosamine	3.05	3.09	3.23
(mmol 100g albumin ⁻¹)	(2.55-3.78)	(2.63-3.58)	(2.79-3.56)
Systolic blood pressure	122	137**	141 ²²²
(mm Hg)	(112-142)	(118-142)	(132-146)
Diastolic blood pressure	79	81	87 ²
(mm Hg)	(72-90)	(80-84)	(80-94)

*, **: p<0.05, p<0.01, group 1 v 2

¹¹: p<0.01, group 3 v 2

^{2,222}: p<0.05, p<0.001, group 3 v 1

TABLE 2. Renal characteristics at baseline

	GROUP		
	1	2	3
AER	3.2	36.2***	321.4 ²²²
($\mu\text{g min}^{-1}$)	(2.1-8.8)	(22.1-95.9)	(201.9-1215.9)
β_2 -M ER	27.9	68.4	33.2
(ng min^{-1})	(6.6-85.2)	(4.0-540.8)	(13.9-209.8)
Serum β_2 -M	1.43	1.81	1.79
(mg l^{-1})	(1.16-1.85)	(1.47-2.01)	(1.48-2.03)
Serum creatinine	89	87	97
($\mu\text{mol l}^{-1}$)	(59-118)	(68-118)	(82-124)

*** $p < 0.001$, group 2 v 1

²²² $p < 0.001$, group 3 v 1 and 2

β_2 -M ER β_2 -microglobulin excretion rate

TABLE 3. Measures of renal function in normoalbuminuric, microalbuminuric and macroalbuminuric IDDM patients after 1 months treatment with bromhexine and placebo.

	GROUP					
	Normoalbuminuric		Microalbuminuric		Macroalbuminuric	
	Placebo	Bromhexine	Placebo	Bromhexine	Placebo	Bromhexine
AER ($\mu\text{g min}^{-1}$)	3.3 (1.9-13.2)	3.6 (1.7-13.5)	37.1 (20.1-103.3)	40.3 (20.7-128.6)	443.4 (292.4-2592.9)	396.9 (247.8-2160.3)
β_2 -MER (ng min^{-1})	27.9 (7.5-51.3)	19.2 (1.4-113.0)	39.3 (10.6-101.2)	43.7 (2.3-200.6)	58.9 (24.7-423.3)	53.4 (31.2-171.8)
Serum $\beta_2\text{M}$ (mg l^{-1})	1.42 (0.91-1.89)	1.65 (1.05-1.98)	1.76 (1.50-1.99)	1.88 (1.60-2.09)	1.96 (1.55-2.96)	1.80 (1.48-2.09)
Serum creatinine ($\mu\text{mol l}^{-1}$)	86 (77-108)	85 (80-103)	91 (78-115)	95 (85-115)	110 (86-180)	96 (81-110)

TABLE 4. Glycaemic control and blood pressure after one months treatment with bromhexine or placebo in the 3 groups of patients.

	GROUP					
	Normoalbuminuric		Microalbuminuric		Macroalbuminuric	
	Placebo	Bromhexine	Placebo	Bromhexine	Placebo	Bromhexine
Fructosamine (mmol 100g albumin ⁻¹)	3.08 (2.55-3.34)	2.95 (2.42-3.30)	3.07 (2.81-3.38)	3.09 (2.63-3.58)	3.36 (3.19-3.60)	3.34 (3.14-3.58)
HbA _{1c} (%)	7.8 (6.1-10.3)	8.3 (6.3-10.2)	10.1 (8.0-11.4)	9.2 (6.3-12.9)	10.3 (9.1-11.7)	9.2 (7.2-10.7)
Systolic BP (mm Hg)	120 (110-138)	121 (104-142)	137 (124-150)	135 (126-142)	141 (138-150)	143 (138-150)
Diastolic BP (mm Hg)	78 (70-92)	78 (70-88)	76 (60-86)	80 (70-86)	86 (76-94)	86 (78-90)

Figure One

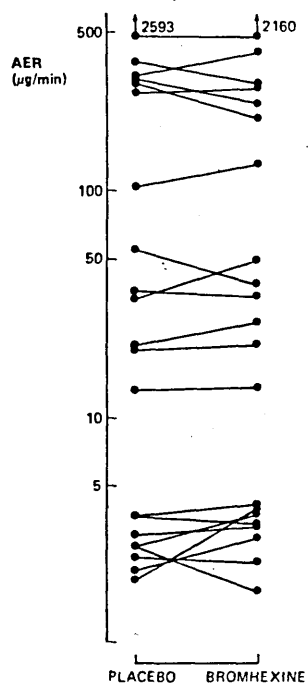


Figure Two

